CHEMICAL REVIEWS

Review

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Gates of Enzymes

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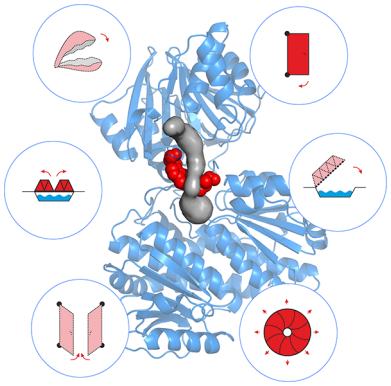

CONTENTS

1. INTRODUCTION

Enzymes are very efficient catalysts that are essential for the functioning of living organisms. The low efficiency of biocatalysts produced de novo relative to those that have evolved naturally demonstrates that our understanding of enzymatic catalysis is still incomplete.[1−4] The dynamic motion of enzymes during catalytic events is one of the many aspects of protein chemistry that are currently insufficiently well understood.[5−9] On one hand, proteins need to have well-defined and organized structures in order to maintain stable functionality in the intracellular environment. On the other hand, some degree of flexibility is often required for catalytic activity. Molecular dynamics simulations have provided key insights into the importance of protein dynamics in catalysis, such as the observation of substrate access and product exit pathways that cannot be identified by inspecting crystal structures.[10] Csermely et al. recently reported that mutations in regions that affect protein dynamics, such as hinge regions that are important in substrate binding, can have dramatic effects on catalytic activity.[11] In this review, we highlight the role of protein gates as another class of highly dynamic structures that play key roles in protein function.

Given the importance of gates for enzymatic catalysis, the number of studies that have examined them systematically is surprisingly small. Conformational gating in proteins was first described by McCammon and co-workers in 1981, but there have been relatively few systematic studies in this area since then.[12−14] Moreover, much of the available data on gates in macromolecular systems is hidden or otherwise dispersed within the scientific literature, partly because there is currently no consensus regarding what defines a gate. Some authors describe all residues that affect the ligand's access to a target area as gating residues, whereas others apply the term exclusively to structural features that undergo large movements during the gating event. In this review, we define a gate as a dynamic system consisting of individual residues, loops, secondary structure elements, or domains that can reversibly switch between open and closed conformations and thereby control the passage of small molecules—substrates, products, ions, and solvent molecules—into and out of the protein structure. Under this definition, the anchoring residues that stabilize the open or closed conformations of a gate are not themselves gating residues. However, because of their various interactions with the gating residues, they can control the size and properties of the ligands that pass through the gate as well as the frequency of the exchange events.

Gates can be found in various systems, including enzymes, ion channels, protein−protein complexes, and protein−nucleic acid complexes.[14,15] In this work, we focus specifically on gates in enzymes. We attempt to answer three basic questions—why, how, where—by describing the molecular function, structural

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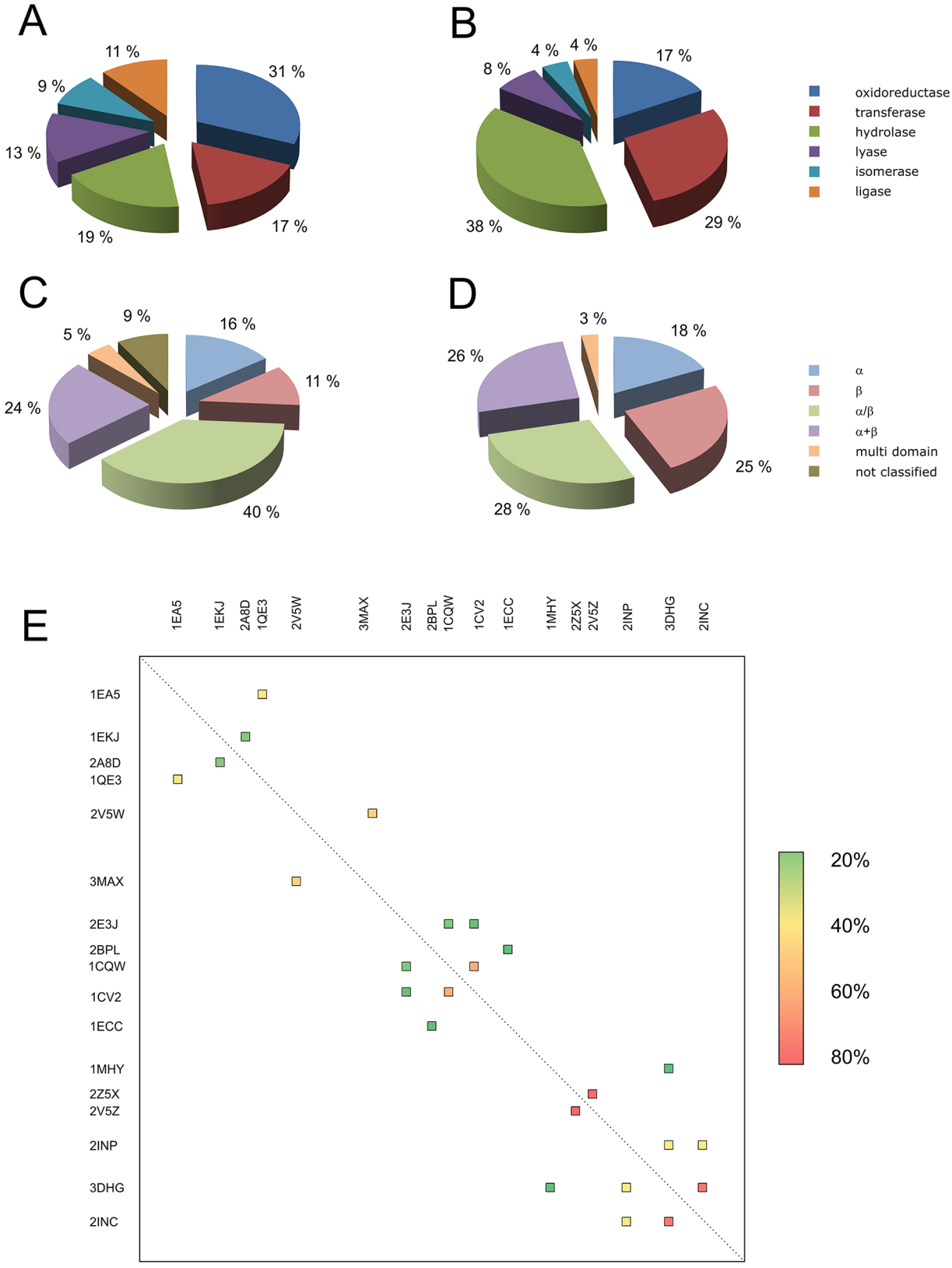

Figure 1. Distribution of (A) reviewed proteins and (B) proteins from the PDB database according to EC classes, (C) reviewed proteins and (D) proteins from the PDB database according to SCOP classification, and (E) identity matrix of reviewed enzymes (only the enzymes with sequence identities above 20% are shown for clarity).

basis, and location of gates within protein structures. We discuss 71 illustrative examples of enzymes that together contain 129 different molecular gates and propose a system for their classification. Reviewed enzymes were chosen based on a literature search with a set of keywords corresponding to gates and conformational changes in enzymes. A preliminary set of

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protein structures was filtered out, leaving only those entries for which both open and closed conformations were described. The final set represents different classes of enzymes (Figure 1A) and different protein folds (Figure 1C) and spans structurally and functionally the entire enzyme world. Among 71 chosen enzymes, only 17 have higher than 20% sequence identity with other set members (Figure 1E). The proposed classification system provides a useful framework for comparing gates of different enzymes and drawing general conclusions about gate function, structure, and position. Moreover, the classification scheme is easily extendable to describe the new gate types that will almost certainly be revealed by structural and functional analyses of newly isolated enzymes in the future.

2. MOLECULAR FUNCTION OF GATES

Analyses of protein dynamics have identified a number of enzymes with gates, suggesting that these structures are rather common. What is the molecular function of the gates? It seems that in enzymes they facilitate precise control over processes that are directly linked to catalysis. Enzyme gates can (i) contribute to enzyme selectivity by controlling substrate access to the active site, (ii) prevent solvent access to specific regions of the protein, and (iii) synchronize processes occurring in distant parts of the protein (Figure 2). The proper function of

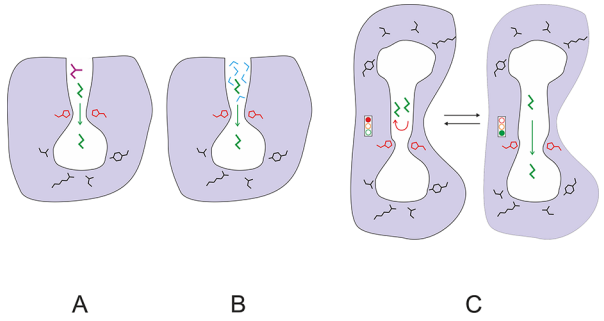

Figure 2. Schematic illustration of the molecular functions of protein gates: (A) control of substrate access, (B) control of solvent access, (C) control and synchronization of reactions. Protein is represented by the area colored in gray, active site cavity by the area in white, gating residues by red lines, substrate molecules by green or violet lines, and water molecules by blue lines.

even the simplest gates can potentially be essential for catalysis, and the gating event may even represent the rate-limiting step of the catalytic cycle. Interestingly, different gating residues within a single protein molecule may be responsible for restricting the access of specific substrates. High variability of the gating residues within an enzyme scaffold can lead to the evolution of enzyme families whose members are selective for specific substrate types. The best known example of such specialization within a single enzyme family is provided by the cytochromes P450.[16]

2.1. Control of Substrate Access

Enzyme selectivity has been traditionally explained by the "lock and key" model,[17] which was subsequently complemented by the "induced fit" or "hand in glove",[18] "selected fit",[19] and "keyhole, lock, and key"[20] models. In many cases, these models provide an adequate description of enzyme selectivity based on adjustable complementarity between the active site and the cognate substrates. However, research conducted over the past decade has shown that regions located further from the active site can also affect enzyme selectivity. Substrate access pathways, which often incorporate molecular gates, impose additional constraints on ligand binding to the active site.[20] The ability of ligands to traverse these access pathways can be controlled by (i) size discrimination at the narrowest point along the pathway forming a bottleneck, (ii) geometrical constraints, e.g., the curvature of the pathway, and (iii) specific molecular interactions such as hydrogen bonds, electrostatic interactions, and hydrophobic interactions with the residues comprising the access pathway. Protein gates can be regarded as molecular filters that discriminate between molecules as similar as molecular oxygen and carbon monoxide in NiFe hydrogenases[21,22] or water and hydroxyperoxide in catalases.[23,24] Gates act as filters in a wide range of enzymes, controlling the range of substrates that can be accepted by broad-specificity cytochromes P450,[25] the stereospecificity of epoxide hydrolases,[26] and product length in undecaprenyl-pyrophosphate synthases.[27]

One of the first systematic descriptions of the influence of the gating process on substrate binding was reported by Szabo et al., who assumed that the switching between the open and the closed conformations of the gate was a stochastic process.[28−30] This model was successfully used to demonstrate that despite conformation gating[13] acetylcholinesterase can bind acetylcholine with a rate constant of 10^9 M^{-1} s^{-1} and predict the rate of formation of the enzyme−substrate complex in choline oxidase.[31] Since gates create a barrier on the substrate access pathway, the kinetic rate constant for passage over the barrier can be obtained using Kramers' reaction rate theory or its later modifications.[32−34] This methodology was used to compare the results of computational and experimental studies on the passage of the tetramethylammonium cation through acetylcholinesterase[35] and migration of ammonia through carbamoyl phosphate synthetase.[36]

2.2. Control of Solvent Access

Spatial localization of the hydrophobic and hydrophilic regions within the structure of a protein is important in maintaining its proper fold and can also be crucial for catalytic function. The various steps of an enzymatic reaction may require different environments. These distinct environments can be generated by having the individual steps occur in spatially separate regions of the protein, but this does not eliminate the problem of transporting the substrate between these sites. There are important problems to be addressed, including transporting polar molecules from a polar environment to a nonpolar one and separating hydrophilic compartments from hydrophobic ones within the structure of a single protein.

In some proteins, these problems are addressed by the presence of selective barriers that permit passage of solutes but not water molecules. Crystallographic and NMR data can be used to identify cavities within a protein structure accessible to water molecules. Exclusion of water from some parts of the cavity, such as the active site or a specific tunnel, is essential for functioning of numerous enzymes. In simple cases, the gates may prevent the entrance of water molecules into the cavity when a substrate or a cofactor is not present, as occurs in rabbit 20a-hydroxysteroid dehydrogenase.[37] In more complex cases, the gates may permit access only to a specific part of the cavity, as occurs in carbamoyl phosphate synthetase[36] and imidazole glycerol phosphate synthase.[38] In the cytochromes P450, a "water channel" controls hydration of the substrate in the active site, which is extremely important for cytochrome activity.[16]

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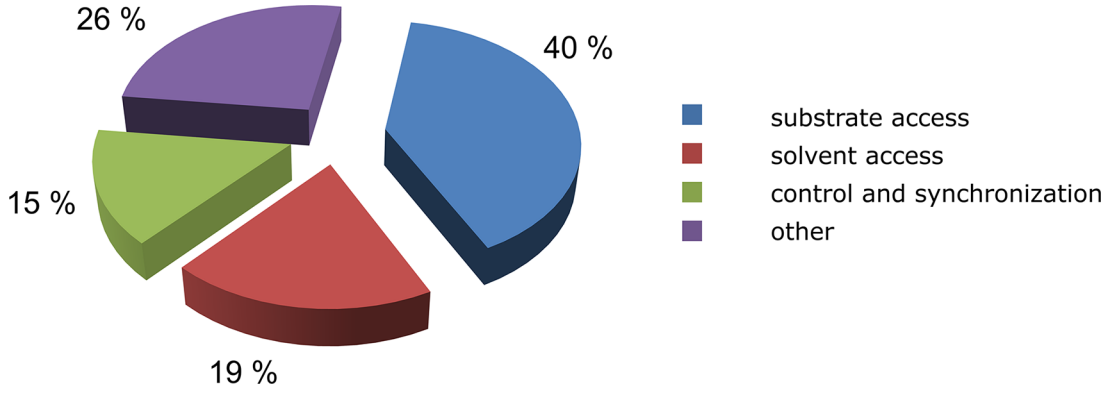

Figure 3. Distribution of gate functions; 71 proteins with 129 different gates were analyzed. Percentages shown in the figure are based on all of the identified functions of each gate; individual gate may perform multiple functions. Detailed description of the analyzed proteins is provided in Table 3.

The potential importance of gates that act as solvent barriers is further illustrated by the example of enzymes with ammonia tunnels. In these proteins, gates prevent water from entering the channel and protonating the ammonia, which is essential for maintaining its nucleophilic character.[39]

Control of water access can be seen as a special case of the function described in section 2.1. However, when discussing water exclusion, the main emphasis is on the water permeability of the gates and their ability to distinguish water molecules from other ligands. The gate can simultaneously act as a barrier to passage of water molecules while acting as a selective filter for other molecules, allowing them to access the active site. Gates of this type resemble semiselective membranes that can distinguish between species such as water and ammonia, allowing only the second to pass. It is worth noting that passage of 'permitted' species can be facilitated by rearrangement of an individual gating residue, such as K99 in imidazole glycerol phosphate synthase. Conversely, passage of water molecules through gates of this type would often require significant conformational changes in all of the residues that comprise the gate.[38] This makes gates in enzymes far more sophisticated than semipermeable membranes.

2.3. Control and Synchronization of Reactions

Another function of gates becomes apparent when considering enzymes with two or more active sites. Many protein structures contain tunnels to facilitate efficient migration of intermediates and gates to synchronize chemical reactions. Such arrangements can be compared to a pair of workers on an assembly line. The second worker has to be ready before he can receive a product from the first one. Moreover, the products generated by the first worker must satisfy certain standards. The control gates located between the workers regulate the exchange of products over a well-defined period of time. Gates of this kind are common in ammonia-transferring enzymes, suggesting that they are old in evolutionary terms and functionally important.[40−43] The need to efficiently transport ammonia within the interior of the protein may be related to its high cellular toxicity. We speculate that gates of this kind may be present in many enzymes that have multiple active sites connected by internal tunnels for the transport of intermediates. Many such enzymes have been studied in some detail, including carbamoyl phosphate synthetase, which has tunnels for ammonia and carbamate transportation;[36] asparagine synthetase,[44] glucosamine 6-phosphate synthase,[45] and glutamate synthase,[46] all of which have tunnels for ammonia transportation; tryptophan synthase for indole[47] and carbon monoxide dehydrogenase/acetyl coenzyme A synthase for carbon monoxide transportation.[48]

Systematic analysis of the functions of the known gates in the 71 proteins discussed in this article revealed the following distribution of gate types: 40% of the studied gates control substrate access, 19% control solvent access, 15% control and synchronize catalytic events, and 26% have other function (Figure 3).

3. STRUCTURAL BASIS OF GATES

Gates are dynamic systems that can make reversible transitions between open and closed states. They vary in size and complexity, from individual amino acid residues to loops, secondary structure elements, and even domains. The simplest gates consist of only one amino acid side chain that can close or open an access pathway by rotating. Opening and closing of more complicated systems can involve the synchronized movement of two or more residues, and the largest systems involve rearrangements of secondary elements or even entire domains (Table 1). For larger systems, movement of the gate may cause formation of a tunnel or enclosed cavity in addition to permitting or denying access to selected species.[49,50] The following parameters can be useful for describing and discriminating between gates: (i) their constituent residues, (ii) their anchoring residues, (iii) the hinge region, i.e., the amino acids that make the structure flexible and allow it to move, (iv) the gate's position, (v) the gate's bottleneck diameter in the open and closed states, (vi) changes in the bottleneck's size over time, (vii) the energy required to switch the gate from one state to the other, and (viii) the energy required for passage of specific molecules through the gate.

3.1. Residue Motion: Wings

The energetic barriers for residue rotation are quite small, 1−16 kcal/mol.[51] While generally low, such barriers can nevertheless be large enough to significantly affect the probability that a given species will be able to pass through the gate or the rate at which they do so. Depending on the particular amino acid and its surroundings, one or both states of the gate may be stabilized by interactions with anchoring residues, e.g., hydrophobic interactions, H bonds, ionic interactions, salt bridges, and $\pi-\pi$ interactions. The strongest effect on the control of the passage is achieved when a large gating residue is located in the bottleneck of the pathway. The most common residues in this role are those whose side chains contain aromatic rings, i.e., W, F, and Y (Figure 4). Wing-type gates are common and can be found in enzymes such as imidazole

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Table 1. Classifying Enzyme Gates According to Their Structural Basis

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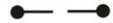
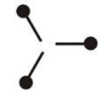
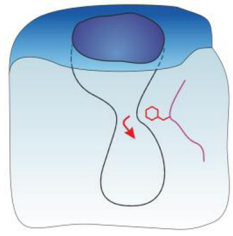
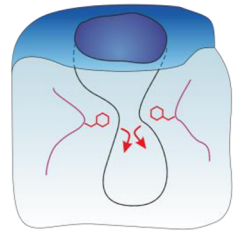
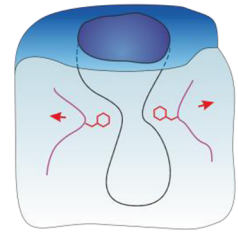
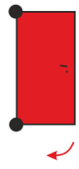
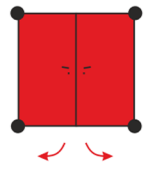
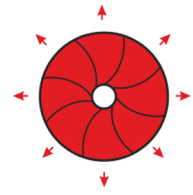
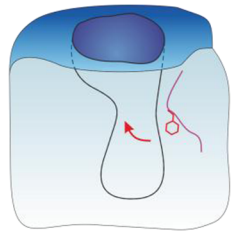
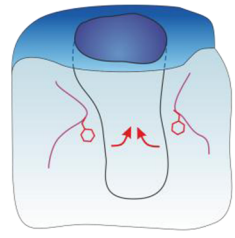
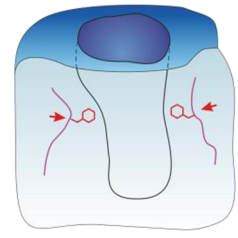
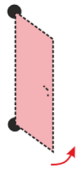
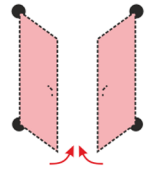
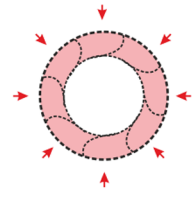
Symbol			
Scheme of closed state			
Illustration of closed state			
Scheme of open state			
Illustration of open state			
Metaphor	wing	swinging door	aperture
Mechanism and moving part	side chain rotation	side chain rotation	backbone motion
Structural basis	1 residue	2 residues distant in sequence	2-4 residues distant in sequence
Amplitude of motion	< 2Å	< 3Å	< 3Å
Time scale	ps – μs	ps – μs	ns – μs
Other features	anchoring residue	anchoring residues	hinge and anchoring free

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Table 1. continued

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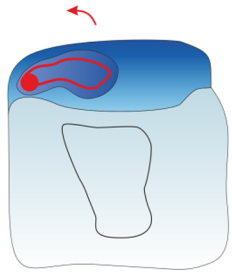
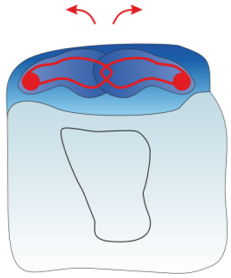
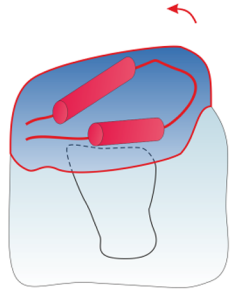
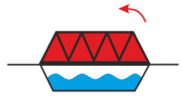
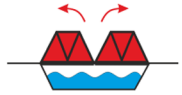
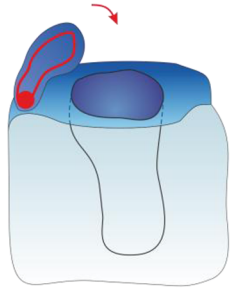
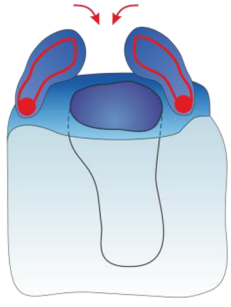
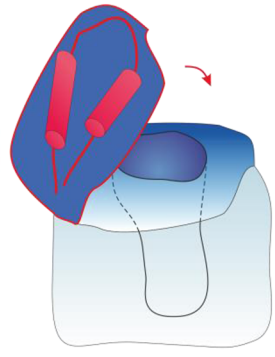
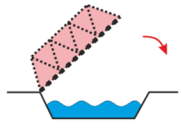
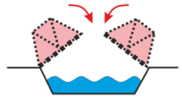
Scheme of closed state			
Illustration of closed state			
Scheme of open state			
Illustration of open state			
Metaphor	drawbridge	double drawbridge	shell
Mechanism and moving part	fragment movement	fragments movement	domain movement
Structural basis	~8-20 residues neighbour in sequence	2x ~8-20 residues neighbour in sequence	domain
Amplitude of motion	> 5Å	> 5Å	> 5Å
Time scale	ns – μs	ns – μs	ms – s
Other features	hinge region, anchoring residues	hinge region, anchoring residues	hinge region, anchoring residues

glycerol phosphate synthase,[38] cytidine triphosphate synthetase,[52] methane monooxygenase hydroxylase,[53] FabZ β-hydroxyacyl-acyl carrier protein dehydratase,[54] and cytochrome P450.[25,55] Even small gates of this type may require an activating agent to open. For example, the gate in the water channel of human monooxygenase CYP3A4 is created by the

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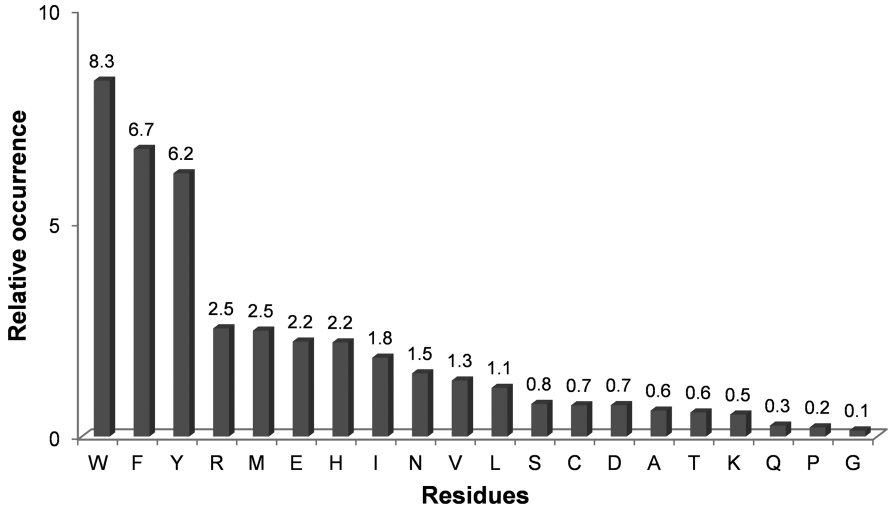

Figure 4. Relative occurrences of specific amino acid residues in wing and swinging door gates; 71 proteins with 129 gates were analyzed, and 154 residues that form wings or swinging doors were identified. Detailed description of the analyzed proteins is provided in Table 3. Values were normalized against the frequency with which each amino acid appears in all of the protein structures of the UniProtKB/Swiss-Prot database (2012_07).

interaction of the conserved residue R375 with the heme, which opens upon cytochrome P450 reductase binding to the enzyme.[56]

3.2. Residue Motion: Swinging Doors

A more complex type of gate consists of two residues that can rotate but are stabilized in the closed conformation by a mutual interaction. Lario et al. introduced the phrase "swinging door" to describe gates of this type that were identified in cholesterol oxidase type I.[57] Some swinging door gates open by having both residues rotate in the same direction, while in others the two residues rotate in opposite directions. Common stabilizing interactions in swinging door gates include π stacking as occurs in the F−F pair of cytochrome $P450_{3A4}$[58,59] and acetylcholinesterase,[60] ionic interactions as in toluene-4-monooxygenase[61] and cytochrome $P450_{cam}$, $P450_{BM3}$, and $P450_{eryF}$,[25,55] aliphatic hydrophobic interactions such as those between the F−I, the F−V, and the F−L pairs of cytochrome $P450_{3A4}$,[58,59] aliphatic interactions such as those between the R−L and the L−I pairs of cytochrome $P450_{3A4}$,[58,59] and hydrogen bonds such as that between the R−S pair in cytochrome $P450_{3A4}$.[58] The open conformations of one or both of the gate residues may also be anchored, depending on the amino acids surrounding the gate. In comparison to wing gates, gates consisting of two residues can control wider tunnels and channels. It is worth mentioning that the individual residues that comprise a swinging door gate may simultaneously be components of another gate, as occurs in cytochrome $P450_{3A4}$.[58] Literature data indicate that most gates of this type consist of F−F pairs, and one way to screen for potential gates is to search for phenylalanine sandwiches.

3.3. Residue Motion: Apertures

Proteins undergo low-frequency breathing motions that may involve synchronized movements of bottleneck residues. In contrast to the previously described gates, the residues that form aperture type gates do not need to rotate and can maintain a rigid conformation. Their movements occur as a result of the synchronized relocalization of the enzyme backbone during its breathing motions. The ability of a given species to pass through gates of this type depends on the length of time the gate remains in the open state, which is determined by the enzyme's rigidity (especially in terms of the compartments housing the gating amino acids) and the strength of the interactions between the gating residues. Gates of this kind can therefore switch between states at different frequencies, which can be adjusted by mutating the gating residues. Aperture-type gates have been identified in several enzymes including carbamoyl phosphate synthetase,[36] choline oxidase,[31] glutamate synthases,[62] extradiol dioxygenases-homoprotocatechuate 2,3-dioxygenase,[63] cytochrome $P450_{eryF}$,[25] and acetylcholinesterase.[64]

3.4. Motions of Loops and Secondary Structure Elements: Drawbridges and Double Drawbridges

The movements of loops and secondary structure elements can provide an energetically favorable method of controlling access for larger ligands. The gates described above consist of individual residues and would not provide sufficient control for enzymes that have large substrates and correspondingly large active site cavities. In many cases, the loops involved in access control also contribute to formation of substrate/cofactor binding cavities. Alternatively, in enzymes with complex systems of internal tunnels such as the members of the cytochrome P450 family, the dynamic motion of the protein structure, especially the flexible B−C and F−G loops in the cytochromes P450, plays a vital role in the opening and closing of the tunnels.[16] Protein motions of this type can also merge different tunnels, creating a wider opening. Here, gating elements control the access of large substrates by merging and dividing the space shared by the tunnels.[16] However, in such cases the movements of the loops can cause formation of smaller and more selective gates such as the swinging doors described in the preceding sections.[56,58]

Movements of loops and secondary structure elements can change the solvation of a cavity or the gate itself. The equilibrium between the open and the closed conformations depends on the anchoring residues and the flexibility of the hinge region. All of these elements play important roles in the movements of large gates. The conserved GxG motif found in most cytochrome P450 family members provides a good example.[65] Depending on cytochrome isoform, the motif flanks either one or both ends of the B−C loop. It increases the

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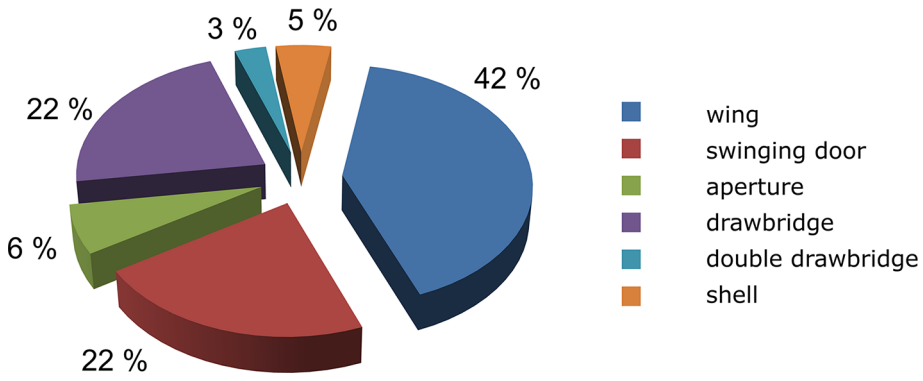

Figure 5. Frequencies of different gate types based on analysis of 71 proteins with 129 gates. Detailed description of the analyzed proteins is provided in Table 3.

flexibility of the loops, lowers the energy required for their motion, and facilitates tunnel opening and closing. The loops' variable lengths and levels of flexibility mean that each member of the P450 family has a gate with unique properties.[16]

3.5. Domain Motion: Shell

Large domain motions in enzymes are not generally regarded as gating systems. "Gates" of this scale are common in ion channels, which are beyond the scope of this review.[14] However, one might expect that such large gates could be present in enzymes that catalyze reactions of very large substrates. Indeed, a gate of this kind has been observed in RNA polymerase, whose clamp domain opens to permit entry of promoter DNA during initiation, closes to establish a tight grip on the DNA during elongation, and then opens again to release the DNA during termination.[66] Interestingly, movement of large domains may protect enzymes from small molecule leakage and control their transport through long tunnel networks. This has been observed in carbon monoxide dehydrogenase/acetyl coenzyme A synthase, which operates as a tetrameric complex of distinct subunits.[67] The cap domain movement of epoxide hydrolase from *Mycobacterium tuberculosis* is another enzyme with a domain-scale gate that controls substrate access to the active site cavity.[68] Monomers of phospholipase A2 control access to their interface and the active site by adopting a different conformation during dimer aggregation.[69] Large domain movements often require an additional source of energy. For example, in the ATP-dependent protease HsIVU,[70] ATP hydrolysis is required to initiate conformational changes and propagate them to the residues that form the gate.

Structures of the gates found in 71 different proteins were analyzed systematically, yielding the results presented in Tables 2 and 3. The most common gate types are wings, swinging doors, and drawbridges, while apertures, double drawbridges, and shells are less common (Figure 5). However, these numbers may be distorted by the difficulty of identifying different gate classes by experimental methods or molecular dynamics simulations. Movements of only a few residues are more easily captured than those of secondary structure elements or domains due to the different time scales involved and the sizes of the moving structures. Moreover, gates may be controlled by overlapping processes that occur on different time scales and affect different structural regions.[50]

4. LOCATIONS OF GATES

The roles of gates in the enzymatic catalysis discussed above suggest that these structures are natural hot spots for modifying enzyme properties. Identification of structural components of natural gates would therefore be very useful to protein designers. This raises a question: how and where should one look for the gates? Gates in proteins can be identified experimentally by protein crystallography and NMR spectroscopy and computationally by molecular dynamics simulations and normal-mode analysis.

Crystallographic analyses can provide information on alternative configurations of particular amino acids in a protein structure. The presence of residues that can adopt both open- and closed-type configurations along the access or release pathways of ligands, ions, and solvents may suggest the presence of a gate. However, in order for a gate to be detected by crystallography, it is necessary for both the open and the closed conformations to be sufficiently represented. Despite this restriction, crystallographic analyses have identified gates in tryptophan synthase,[71] haloalkane dehalogenase LinB,[72] L-amino acid oxidase,[73] and toluene-*o*-xylene monooxygenase.[74] In some cases, only one conformation will be present in the solved crystal structure, which may create a somewhat distorted picture, suggesting the absence of a gate when the open conformation is stabilized or the absence of a pathway when the closed conformation is stabilized.[31,61,75]

The limitations of crystallographic analysis can be overcome by advanced NMR spectroscopy, which makes it possible to study multiple protein conformations simultaneously, over time scales ranging from picoseconds to milliseconds.[7,76] Such analyses provide information on both the open and the closed states as well as the population of each state and rate of their interconversion. NMR techniques have been used to measure the rate of exchange between the open and the closed conformations of triosephophate isomerase,[77,78] HIV-1 protease,[79] and dihydrofolatereductase.[80,81] Overall, the utility of NMR for studying distant effects of mutations on protein dynamics suggests that it has great potential for investigating gating mechanisms.[9]

Some of the most useful tools for identifying gates are the computer programs developed for detecting tunnels, channels, and cavities in protein structures.[82] The outputs of CAVER,[83] MOLE,[84] and MOLAXIS[85] can be analyzed to detect bottleneck residues that form a potential gate or identify the best position for introduction of a new gate. Mutations at these "hot spots" can provide enzymes with new selectivities or

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Table 2. List of Enzymes Possessing Gates Described in the Scientific Literature with Indication of Their Function, Structural Basis, and Location

		Function					Structural basis							Location			
No.	Enzyme name	Substrate accessibility	Solvent accessibility	Synchronization of reactions	Other							Active site entrance and active site	Tunnel entrance and tunnel bottleneck	Cofactor cavity	Other
01	2-Amino-2-Desoxyisochorismate Synthase PhzE	+		+		+									+
02	3-Hydroxybenzoate Hydroxylase MHBH		+		+	+!								+	
03	4-Hydroxy-2-Ketovalerate Aldolase DmpG / Acylating Acetaldehyde Dehydrogenase DmpF	+		+	+	+	+						+		
04	4-Hydroxybenzoate Hydroxylase PHBH		+			+!					+			+	+
05	Acetylcholinesterase AChE	+	+			+	+					+			
06	Acylaminoacyl Peptidase				+						+				+
07	α-Amylase TK1436	+			+	+						+			
08	Asparagine Synthetase			+	+	+?							+	+	
09	ATP-Dependent Proteases HslVU	+				+!!							+		
10	Carbamoyl Phosphate Synthetase CPS – type II		+	+			+	+					+		
11	Carbon Monoxide Dehydrogenase / Acetyl Coenzyme A Synthase	+		+	+						+				+
12	Carbonic Anhydrase β – type I		+				+					+			
13	Carbonic Anhydrase β – type II	+	+	+		+		+				+	+		
14	Carboxylesterase pnbCE	+			+	+				+		+	+		
15	Catalase CAT-1 and CAT-3	+	+			+		+				+	+		
16	Cellobiohydrolase CEL 7A	+			+				+						+
17	Cellobiose Phosphorylase	+							+						+
18	Chalcone Synthase	+			+		+						+		
19	Chloramphenicol Halogenase CmlS	+							+				+		
20	Cholesterol Oxidase – type I SCHOX	+	+	+	+	+	+						+	+	
21	Cholesterol Oxidase – type II BsChOx	+			+		+								
22	Choline Oxidase	+						+	+?			+	+		
23	Chondroitin AC Lyase		+		+					+		+			+
24	Copper-Containing Amine Oxidase		+			+							+		
25	Cytidine Triphosphate Synthetase CTPS		+	+		+			+		+	+		+	
26	Cytochrome P450 CYP3A4	+	+			+!!	+				+	+			
27	Dihydrofolatereductase				+				+				+		
28	Digeranylgeranylglycerophospholipid Reductase DGGR	+			+				+	+			+	+	
29	Epoxide Hydrolase H37Rv	+								+				+	
30	Epoxide Hydrolase M200	+			+	+?						+			
31	FabZ β-Hydroxyacyl-Acyl Carrier Protein Dehydratase	+			+	+						+			
32	Formiminotransferase-Cyclodeaminase FTCD				+				+					+	
33	Glucosamine 6-Phosphate Synthase GlmS		+	+	+	+	+		+		+	+			
34	Glutamate Synthases GltS	+	+	+	+		+		+		+	+			
35	Glutamine Phosphoribosylpyrophosphate Amidotransferase	+	+	+	+	+			+		+	+			
36	Haloalkane Dehalogenase DhaA	+	+			+	+					+			
37	Haloalkane Dehalogenase LinB	+				+						+			
38	Histone Deacetylase HDAC1 and HDAC2	+				+	+					+			
39	Histone Deacetylase HDAC8		+	+		+						+			
40	HIV-1 Protease	+								+				+	
41	Homoprotocatechuate 2,3-Dioxygenase	+					+	+				+			
42	Hydrogenase FeFe	+						+						+	
43	Hydrogenase NiFe				+			+!			+				
44	Imidazole Glycerol Phosphate Synthase IGPS		+	+		+		+				+			
45	Inosine 5'- Monophosphate Dehydrogenase				+				+		+				
46	Ketoacyl Synthase KS	+	+			+					+	+			
47	L-Amino Acid Oxidase	+			+	+						+			
48	Lipase B	+			+	+								+	
49	Lon Protease	+							+			+			
50	Mannitol 2-Dehydrogenase				+	+						+			
51	Methane Monooxygenase Hydroxylase MMOH	+		+	+	+	+				+	+			
52	Monoamine Oxidase A	+							+		+				
53	Monoamine Oxidase B	+			+		+		+		+			+	
54	Monooxygenase ActVAOrf6	+	+		+	+			+		+			+	
55	NADH Oxidase				+	+!					+				
56	O-Acetylserine Sulfhydrylase Cysteine Synthase	+				+			+			+			
57	Oxidosqualene Cyclase SceOSC	+				+						+			
58	Phenol Hydroxylase PHHY		+		+		+	+				+	+		+
59	Phospholipase A2		+								+				+
60	Phosphatidylinositol-Specific Phospholipase C		+							+		+			
61	Quercetin 2,3-Dioxygenase		+				+	+					+		

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Table 2. continued

62	Rabbit 20a-Hydroxysteroid Dehydrogenase		+		+				+			+		+
63	Raucaffricine O-β-Dglucosidaseglucosidase	+				+						+		
64	RNA-Dependent RNA Polymerase				+				+					+
65	RNA Polymerase	+									+		+	
66	Toluene-4-Monooxygenase T4MO	+	+		+	+	+					+	+	
67	Toluene-Ortho-Xylene Monooxygenase ToMO	+				+						+	+	
68	Triosephophate Isomerase		+						+			+		
69	tRNA-Dependent Amidotransferase GatDE and GatCAB		+	+	+		+		+			+	+	
70	Tryptophan Synthase		+	+	+	+	+		+			+	+	+
71	Undecaprenyl-Pyrophosphate Synthase				+				+				+	

$+^{!}$ Indicates gates interacting with cofactor. $+^{!!}$ Cofactor assisted gating. $+^{?}$ Classification uncertain.

activities.[86] Zawaira et al.[59] used the CAVER software together with the Protein Interaction Calculator[87] for identifying gating residues within the cytochrome P450 family.

MD simulations are well suited for identification and analysis of gates and their behavior over time. Detailed descriptions of MD methods and their applications in simulating ligand migration can be found in recent reviews.[9,88] Movements of large protein fragments on microsecond time scales can be investigated using Brownian dynamics,[31,89] while Random Expulsion Molecular Dynamics and Steered Molecular Dynamics can be used to study pathways dedicated to transport of specific ligands.[58,90] Some proteins have multiple pathways, each of which accommodates a different ligand or ligand class. This may in fact be a lot more common than is currently realized and can dramatically increase the complexity of gating systems arising from protein movement and the difficulty of identifying the true gating residues. For example, different residues control the ability of inhibitors E2020 and Huperzine A to access the active site of *Torpedo californica* acetylcholinesterase.[60] Similarly, in cytochrome $P450_{3A4}$, different residues in the same tunnel control access of temazepan and testosterone-6OH.[58] The importance of a gating residue identified by computational methods can be confirmed experimentally by site-directed mutagenesis and kinetic experiments.

Studies using the experimental and theoretical approaches for gate identification discussed above have demonstrated that their locations within the protein can vary widely. Gates have been observed (i) at the entry to the active site or even directly inside the active site, (ii) at the entry or in the bottleneck of the protein tunnel connecting the buried active site to the protein surface or connecting two active site cavities, and (iii) at the interface of the cofactor and active site cavities (Figure 6).

4.1. Active Site Entrance and Active Site

The entrance to the active site cavity is a suitable location for a gate, and gates situated here can have strong effects on enzyme activity. In some cases, the gating residues may even be a part of the active site.[91] The simplest gates serve as filters that discriminate between potential substrates and thus play an important role in controlling enzyme selectivity. More advanced systems can prevent substrate entry when the active site residues are not properly oriented, e.g., in enzymes that require conformational changes before substrate binding. Many enzymes have gates at the entrance to their active sites, including acetylcholinesterase,[60] imidazole glycerol phosphate synthase,[38] glutamate synthase,[46] toluene-*o*-xylene monooxygenase,[91] monooxygenase,[92] choline oxidase,[31] NiFe hydrogenases,[21] carbonic anhydrases,[93] formiminotransferase-cyclodeaminase,[94] type III polyketide synthases,[95] and FabZ β-hydroxyacyl−acyl carrier protein dehydratase.[54]

4.2. Tunnel Entrance and Tunnel Bottleneck

The ability of ligands and solvent molecules to move from the media surrounding the protein to the active site can be controlled by gates located at any point along the tunnel. Gating residues may be situated at the tunnel entrance. However, it is more common to find them at the tunnel bottleneck. The tunnel entrance refers to the first shell of residues that define the tunnel and have contact with the bulk solvent. The tunnel bottleneck refers to the narrowest part that can be positioned anywhere along the tunnel (Figure 6). Even a single large residue whose side chain can project into the interior of the tunnel can exert efficient control over the access pathway. One might speculate that it might be favorable to have gates located inside tunnels because this allows their position to be more tightly controlled; their movements are restricted by the surrounding residues, and both the open and the closed conformations can be stabilized via interactions with neighboring amino acids. In contrast, residues located on the surface of the protein possess more degrees of freedom, and it is rare for both the open and the closed conformations to be stabilized. Examples of such gates inside the tunnels can be found in cholesterol oxidase type I,[57] toluene-4-monooxygenase,[61] undecaprenyl-pyrophosphate synthase,[27] homoprotocatechuate 2,3-dioxygenase,[63] 4-hydroxy-2-ketovalerate aldolase/acylating acetaldehyde dehydrogenase,[96] epoxide hydrolase from *Aspergillus niger* M200,[26] and FabZ β-hydroxyacyl-acyl carrier protein dehydratase.[54] Similarly, gates can be situated in the bottlenecks of tunnels connecting two active sites. Gates in such positions are essential for enzymes that catalyze two reactions requiring different environments, such as glucosamine 6 phosphate synthase,[45] imidazole glycerol phosphate synthase,[38] cytidine triphosphate synthetase,[52] carbamoyl phosphate synthetase,[36] and glutamate synthases.[46]

4.3. Cofactor Cavity

Gates can be positioned at the interface of the active site and the cofactor cavity, allowing for more fine-grained control during the reaction. In NADH oxidase, the W47 residue acts as a gate that controls the accessibility of the FAD flavin ring and thus plays a crucial role during the catalytic cycle. The closed conformation is stabilized by hydrogen bonds between the cofactor and the peptide backbone, whereas stabilization of the open form may be advantageous during the initial steps of

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Table 3. Detailed Description of Enzymes Possessing Gates Presented in the Scientific Literature

No.	**Enzyme name**	EC number

Gate function
Information about gate function

Gate location
Information about gate location

Gate structural basis

Schematic drawing of gate class

***?** in front of a picture indicates a lack of data – classification has been made based on residues localisation*

GATE 1- *font colour corresponds to the colour of residues on the picture*

Information about residues, open and closed conformation and mechanism of the changes of gate states

? GATE1 - ? *indicates a hypothetical gate, some data missing to fulfil all requirements, e.g., lack of information about open and close conformation*

*** GATE1- *** *indicates important residues used for gates engineering*

Gate engineering
Information about mutants that change gate state or create new gates, including information about mutants closing and opening tunnels

PDB: *Wild-type (WT) or mutants available in Protein Data Bank database*

References: *All references used for table preparation*

Enzyme function
Information about catalysed reaction

Small picture of whole enzyme with detected tunnels and gates
protein – light blue surface
tunnel – dark blue wire
gates residues – red ball and stick

Large picture – close-up on gate residues
protein – light blue cartoon
tunnel profile – grey spheres
active sites residues (if shown) – green ball and stick
gates residues (elements) – ball and stick representation colour correspond to colour of gate name
cofactor – yellow ball and stick
substrate – yellow ball and stick

All pictures present results of CAVER 3.0 calculations of tunnels visualized by PyMOL. ***Tunnels corresponding to those reported in literature have been visualised.*** *Reader should be aware that calculations were performed using available static structures from the PDB database, therefore calculated tunnels can differ from the tunnels described on the base of results from MD simulations.*

Tunnel	*Name or part of the tunnel*
Length	*Tunnel length in [Å]*
Bottleneck	*Bottleneck diameter in [Å]; data taken from the literature*
Role	*Information about transported molecules*

01	**2-Amino-2-Desoxyisochorismate Synthase PhzE**	4.1.3.27

Gate function
GATE1 – Controls access of ammonia, synchronizes active sites

Gate location
GATE1 – Between the MST (menaquinone, siderophore, tryptophan) domain and GATase1 active site

Gate structural basis

—●

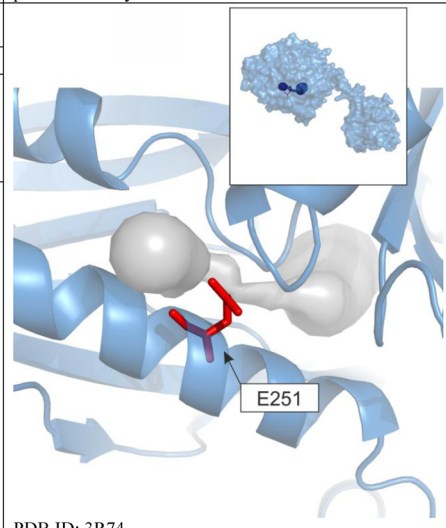
GATE1
E251
forms two hydrogen bonds with N149 in closed conformation
interacts with K254 in open conformation

Gate engineering

PDB: ***WT*** **–** 3R74 (open), 3R75 (closed)

References: [111]

Enzyme function
Utilizes chorismate and glutamine to synthesize 2-amino-2-desoxyisochorismate in the first step of phenazine biosynthesis



PDB ID: 3R74

Tunnel U-shaped	
Length	25
Bottleneck	
Role	Ammonia transport

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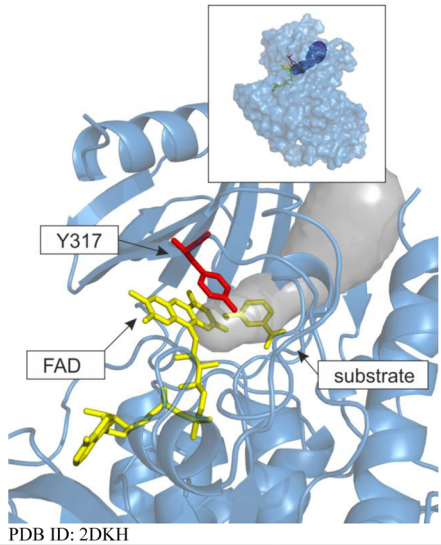
Table 3. continued

02	**3-Hydroxybenzoate Hydroxylase MHBH**	1.14.13.23
Gate function GATE 1 – Controls the contact of NADPH with the isoalloxazine ring, protects FAD from the solvent		**Enzyme function** Conversion of 3-hydroxybenzoate to 3,4-dihydroxybenzoate
Gate location GATE 1 – Between substrate tunnel and the FAD binding pocket		
Gate structural basis		
—●	GATE 1 Y317 The residue creates parallel π–π stacking interaction with FAD Opening – reorientation of both FAD and Y317	
Gate engineering		PDB ID: 2DKH
		Tunnel: E1 / E2 (not shown)
		Length: 20-22 / 32
PDB: *WT* – 2DKH, 2DKI		Bottleneck: 4.5 / 4
References: [98]		Role: O_2 , substrate / Product

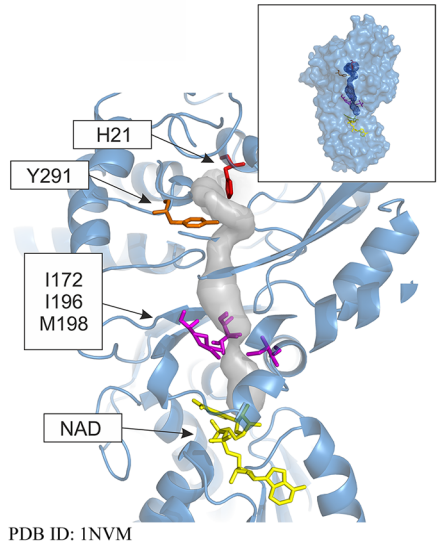
03	**4-Hydroxy-2-Ketovalerate Aldolase DmpG / Acylating Acetaldehyde Dehydrogenase DmpF**	4.1.3.39 / 1.2.1.10
Gate function GATE1 – Controls access of the substrate to the DmpG active site GATE2 – Proton transfer, synchronizing two active sites by controlling the passage of the acetaldehyde GATE3 – Controls intermediate entry to the DmpF active site; facilitates interaction between N171 and NAD		**Enzyme function** Catalyses final two steps in degradation of toxic aromatic intermediates in the meta-cleavage pathway of catechol
Gate location GATE1 – Tunnel entrance in aldolase subunit GATE2 – Tunnel entrance in aldolase subunit GATE3 – Tunnel exit in dehydrogenase subunit		
Gate structural basis		
—●	GATE1 H21 Opening – reorientation of H21	
—●	GATE2 Y291 Opening – reorientation of Y291	
—● ●— —●	GATE3 I172 + I196 and M198 I172, I196 and M198 block the tunnel exit in the dehydrogenase subunit Opening – in the structure with bound NAD^+ – an interaction of N171 with NAD induce unique orientation of I172, additionally I196 and M198 adopt open conformations	
Gate engineering H21A – acetaldehyde and propionaldehyde channelling reduced by more than 70% Y291F – reduced channelling efficiencies by >30% I196L, I196F – no significant changes in the channelling efficiency		PDB ID: 1NVM
		Tunnel between active sites
		Length: 29
PDB: *WT* – 1NVM		Bottleneck: 1.7
References: [96,112]		Role: Acetaldehyde intermediate transport

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Table 3. continued

04	**4-Hydroxybenzoate Hydroxylase PHBH**	1.14.13.2

Gate function
GATE1 – Controls access of the solvent
GATE2 – Controls access of the solvent

Enzyme function
Monooxygenation of *p*-hydroxybenzoate (*p*-OHB) to 3,4-dihydroxybenzoate

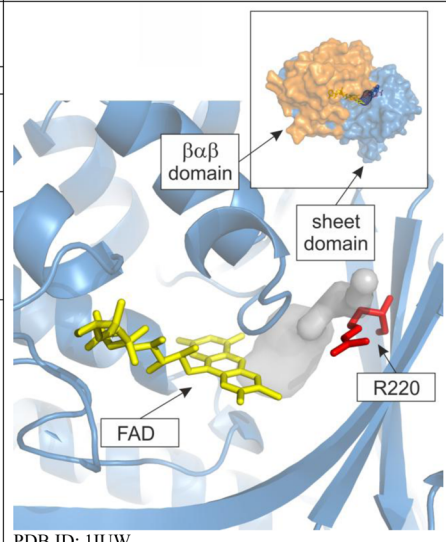
Gate location
GATE1 – Close to the FAD cofactor
GATE2 – One domain of the protein

Gate structural basis

GATE1
R220 and FAD
R220 modulates the dynamics of flavin movements
Opening – reorientation of R220; out – solvent exposed; in – solvent excluded

GATE2
Large domain movement
As substrate (p-OHB) moves forward in the tunnel and reaches its high-affinity site, the βαβ (1-180) and the sheet domains (180-270) are expected to rotate and close the active site onto the substrate

Gate engineering
R220Q – keeps enzyme in the open conformation – loss of selectivity and decrease of activity (100-fold)



PDB ID: 1IUW

Tunnel		
Length		
Bottleneck		
Role	Substrate access	

PDB: ***WT*** **–** 1IUW, ***Mutant R220Q*** **–** 1K0I, 1K0J, 1K0L

References: [113]

05	**Acetylcholinesterase AChE**	3.1.1.7

Gate function
GATE1 – Controls access of the substrate to the active site
GATE2 – Controls escape of the acetic acid and/or the water molecule

Enzyme function
Hydrolysis of acetylcholine

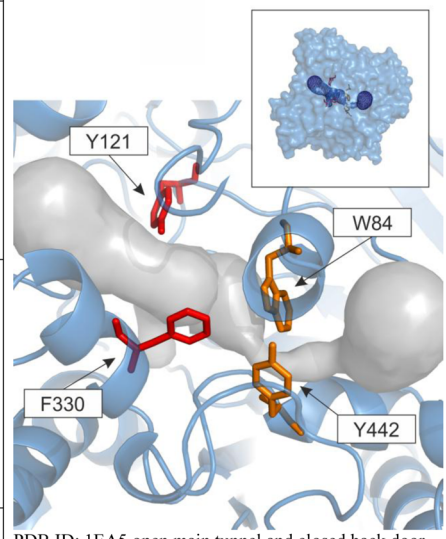
Gate location
GATE1 – Main tunnel – 12 Å from the bottom of the gorge – entrance to the active site
GATE2 – Back door tunnel – on the C67– C94 wall

Gate structural basis

GATE1
F330
F330 controls the entrance of the natural substrate
Second most important residue – Y121, followed by W84, F288, F290, F331,Y334
Cationic substrate (Huperzine A): – movement of F330, Y121 and D72 generate an electrostatic field affecting the substrates
Aromatic substrate (E2020): residues grouped in 3 groups acting as "sender" and "receiver", compose a "conveyer belt" via π- π stacking interactions with benzene ring of E2020:
Group I contains W84, F330, and F331
Group II consists of F288, F290, and Y334
Group III includes Y70, Y121, and W279
In mAChE F338 -Y124

GATE2
W84, G441 and Y442 (TcAChE);W86, G448 and Y449 (mAChE)
Opening - movement of the W84 indole ring, almost 90° rotation to a position where it interacts with Y442
Alternative propositions
?GATE2b –E82, P76, and G77 (with some small movement of D72, E73, and N85)
?GATE2c – Between V71, N85, P86, and M90
?GATE2d – 90° rotation of the F78 and the displacement of V431 and W432
?GATE2e – W84, V129, and G441
?GATE2f – Ω-loop (C67 – C94) with W84 could undergo a flap-like transition
?GATE2g –Facial rearrangement of the loop between W279 and S291

Gate engineering
V129W – 4-fold increase of K_m
V431C – 2-fold increase of K_m



PDB ID: 1EA5 open main tunnel and closed back door

Tunnel	main	back door
Length	20	
Bottleneck	1.2-2.4	1.4-1.7
Role	Acetylcholine	Small molecules

PDB: ***WT*** **–** 1W75, 1ACJ, 1ACL, 1EA5, 2ACE, 1MAH, 1QTI, 1DX6, 1EVE, 1OCE, 2XI4 (open back door)

References: [13,35,60,64,114–123]

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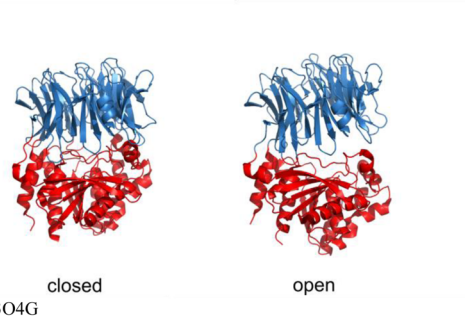
Table 3. continued

06	Acylaminoacyl Peptidase	3.4.19.1
Gate function GATE1 – Controls enzyme activity – only closed form is active		**Enzyme function** Removes acylated amino acid residues from the N terminus of oligopeptides
Gate location GATE1 – Two domains of an enzyme monomer		
Gate structural basis		PDB ID: 3O4G
	GATE1 Two domains may move away to form an opening of about 30°, with D376 being the hinge Open - accept substrate Closed - rearrange active site	Tunnel
Gate engineering		Length
PDB: ***WT*** **–** 3O4G		Bottleneck
References: [124,125]		Role

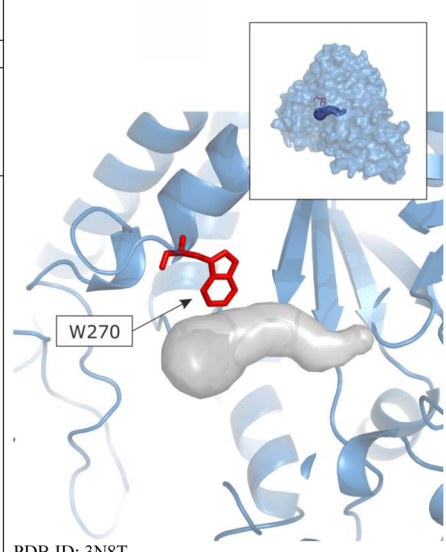
07	α-Amylase TK1436	2.4.1.18
Gate function GATE1 – Controls access of the substrate to the active site, regulates transglycosylation		**Enzyme function** Formation of branch points in glycogen and amylopectin by cleavage of α-1,4 glycosidic bonds and subsequent transfer to a new α-1,6 position
Gate location GATE1 – At the entrance of the active site		
Gate structural basis		PDB ID: 3N8T
	GATE1 W270 The residue displays different conformations depending on the presence or absence of ligands in the active-site pocket Other possible gate-keepers W28, W407, W416	Tunnel
Gate engineering		Length
PDB: ***WT*** **–** 3N8T, 3N92, 3N98		Bottleneck
References: [126]		Role: Substrate recognition and binding

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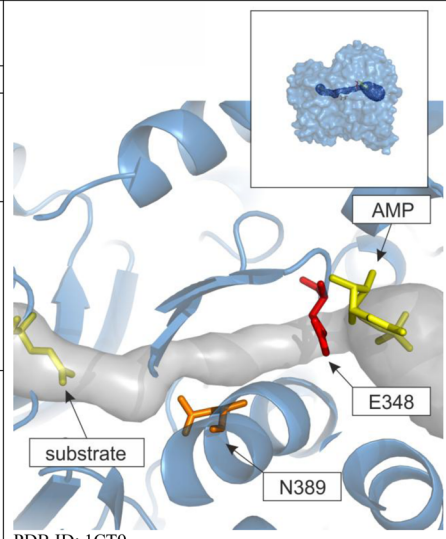
Table 3. continued

08	**Asparagine Synthetase**	6.3.5.4
Gate function ? GATE1 – Synchronizing active sites, establish the intramolecular tunnel for ammonia passage ? GATE2		**Enzyme function** ATP dependent synthesis of L-asparagine from L-aspartic acid
Gate location ? GATE1 – C-terminal end of the ammonia tunnel linking the active site near to the ATP moiety ? GATE2 – C-terminal domain		
Gate structural basis		PDB ID: 1CT9
—●	? GATE1 E348	Tunnel
—●	? GATE2 N389	Length
Gate engineering E348D – impairs acyl-adenylate formation, tunnel is more solvent exposed leading to loss of the ammonia because of an impaired rate of βAspAMP formation		Bottleneck
PDB: ***WT*** **–** 1CT9		Role: Ammonia transport
References: [42,44,127,128]		

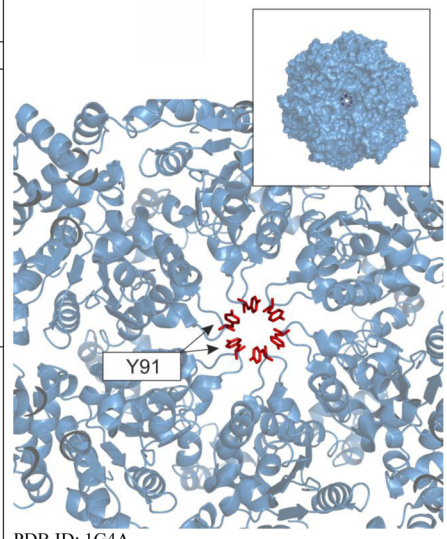
09	**ATP-Dependent Protease HslVU**	3.4.25.2
Gate function GATE1 – Controls access of the substrate to the active site located inside the chamber		**Enzyme function** Degradation of the majority of proteins in a cell
Gate location GATE1 – Translocation tunnel		
Gate structural basis		PDB ID: 1G4A
—●	GATE1 Y91 "Twist-and-open" mechanism - conformational changes induced by ATP hydrolysis are propagated to the gating sequence Y91 can move (180° rotation) from inside HslU toward HslV through the pore – closed pore has diameter 4.4Å open pore has diameter of 19.3Å HslVU works as a hexamer therefore pore diameter depends on the number of Y91 pointed toward HslV	Tunnel
Gate engineering Y91F, V92I, V92A, and V92S – decrease in protein degradation activity G90P, G93P, G90A, G93P, Y91I, Y91C, Y91S, Y91A, V92F, V92C – are not capable to support the proteolytic activity of HslV		Length: -
PDB: ***WT*** **–** 1G4A, 1G4B		Bottleneck: Closed - 4.4; open up to 19.3
References: [13,129,130]		Role: -

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Table 3. continued

10	**Carbamoyl Phosphate Synthetase CPS – type II**	6.3.5.5

Gate function
GATE1 – Desolvation of ammonia
GATE2 – Controls entry of carbamate into the tunnel prior to phosphorylation to carbamoyl phosphate
GATE3 – Controls entry of carbamate into the tunnel prior to phosphorylation to carbamoyl phosphate

Enzyme function
Synthesis of carbamoyl phosphate

Gate location
GATE1 – Large subunit/ammonia tunnel
GATE2 – Carbamate tunnel near the carboxy phosphate binding site
GATE3 – Carbamate tunnel near the site for the synthesis of carbamoyl phosphate

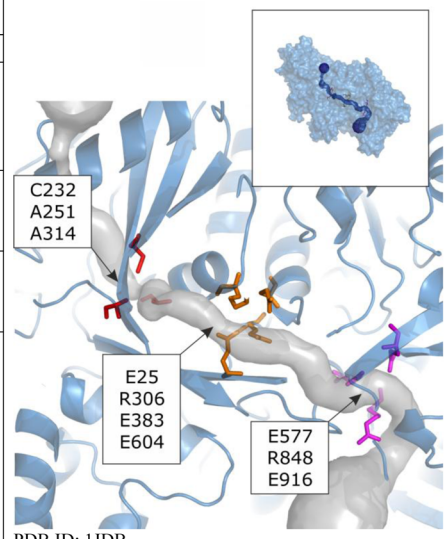
Gate structural basis

GATE1
C232, A251, A314 (barrier 7.2 kcal/mol)
Two more triad that may act as gate/switches for an ammonia passage are:
I234-T249-L310
E217-T244-S307

GATE2
R306, E25, ?E383, ?E604
R306 – ion pair with E25

GATE3
R848, E577 and ?E916
R848 – ion pair with E577

Gate engineering
C232V/A251V/A314V – closing ammonia tunnel - unable to synthesize carbamoyl phosphate using glutamine as a nitrogen source
G359F, G359Y – decoupling separate chemical reactions via creation an escape route for the ammonia intermediate
αP360A/αH361A/βR265A – unable to utilize glutamine for the synthesis of carbamoyl phosphate via creation of an escape route for the ammonia intermediate; full catalytic activity with external ammonia source
E916Q – 10-fold decrease in the rate of carbamoyl phosphate synthesis
E25Q/E383Q – carbamoyl phosphate synthetase activity was diminished 50-fold
E25Q/E383Q/E604Q – glutaminase activity is decreased about 5-fold, and the bicarbonate-dependent ATPase activity is diminished at least 20-fold
E577Q –decrease of carbamoyl phosphate synthesis (100-fold)



PDB ID: 1JDB

Tunnel	Whole	I part	II part
Length	>100	45	
Bottleneck		3.2	
Role	Connects all 3 active sites	Ammonia transport	Carbamate transport

PDB: ***WT*** **–** 1BXR, 1JDB
References: [36,41,103,131−134]

11	**Carbon Monoxide Dehydrogenase / Acetyl Coenzyme A Synthase**	1.2.7.4 / 1.2.99.2 / 2.3.1.169

Gate function
GATE1 – Controls the reaction, protects from CO leakage and controls CO access to Ni_p-Ni_d-[Fe_4-S_4] cluster (A cluster – acetyl-CoA synthase active site)

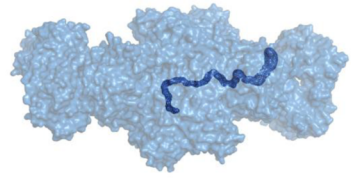
Enzyme function
α2β2 tetramer; β subunit catalyses the reduction of CO_2 to CO (C cluster), α subunit is responsible for the synthesis of acetyl-CoA from CO, coenzyme A (CoA) and a methyl group donated by a cobalamin-containing protein (A cluster)

Gate location
GATE1 – 20 Å from the A cluster – on the A and C cluster border

Gate structural basis

GATE1
Large conformation change of subunit α, gating residues – residues of the α subunit N-terminal domain 143-148
Open conformation
– accessible active site
– closed tunnel
– F512 moves to a position within 4 Å of both Ni_p and Ni_d blocking putative ligand binding to the axial coordination site of Ni_p
Close conformation
– closed active site
– open tunnel
– F229 blocks axial ligand binding to Ni_d but not to the Zn ion

Gate engineering
A110C, A222L, A265M – block the tunnel between the A and C-clusters; A222L complete blocking
A578C, L215F, A219F – block the tunnel between the C clusters
F70W, N101Q – block a region at the ββ subunit interface that might dynamically connect the tunnel with a newly discovered water tunnel



PDB ID: 1OAO
Only one from two symmetrical tunnels is shown on a picture.

Tunnel	
Length	130 between A clusters, additional two tunnels connecting C clusters 37 + additional water channel
Bottleneck	
Role	CO transport between two active sites

PDB: ***WT (M. thermoacetica)*** **–** 1OAO (open+closed form), ***WT*** **(*C. hydrogenoformans*) –** 1RU3 (closed form)
References: [48,67,135−137]

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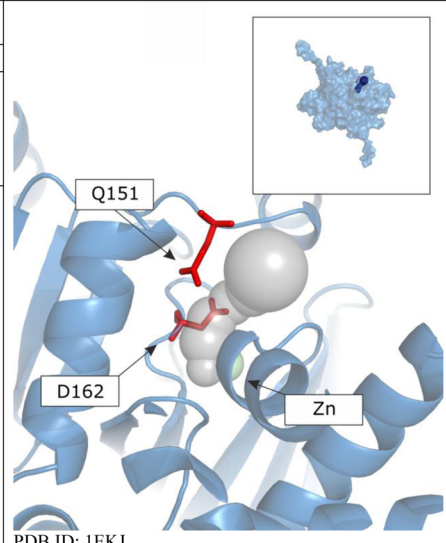
Table 3. continued

12	**Carbonic Anhydrase β – type I**	4.2.1.1
Gate function ?GATE1 – Protects zinc cation site		**Enzyme function** Catalyses a reversible reaction to form bicarbonate from carbon dioxide and water
Gate location ?GATE1 – Vicinity of the active site		 PDB ID: 1EKJ
Gate structural basis		
?	?GATE1 D162 donates a hydrogen bond to Q151 Acts as a gatekeeper residue by excluding anions from the zinc ligand environment that cannot donate a hydrogen bond at this position	
Gate engineering		
PDB: ***WT*** **–** 1EKJ		Tunnel
		Length
		Bottleneck
References: [93]		Role: Acetate ion, CO_2 transport

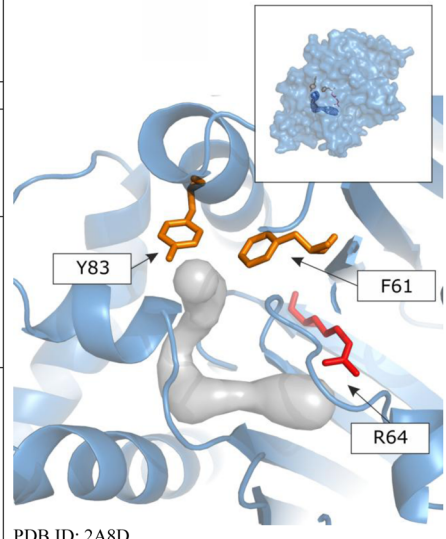
13	**Carbonic Anhydrase β – type II**	4.2.1.1
Gate function GATE1 – Controls access of the bicarbonate ion GATE2 – Controls access of the solvent, protects active site, controls transport of bicarbonate ion from the active site or from solution to the allosteric pocket		**Enzyme function** Catalyses a reversible reaction to form bicarbonate from carbon dioxide and water
Gate location GATE1 – Middle of the tunnel GATE2 – Vicinity of the active site in a narrow hydrophobic active site cleft that lies along the dimer or pseudo-dimer interface		 PDB ID: 2A8D
Gate structural basis		
	GATE1 R64 – HICA Rotation of the guanidinium group allows migration of bicarbonate ion into the allosteric site	
?	GATE2 F61, V62?, Y83, and V87 Other anhydrases PPCA – F422, I173, Y444, Y448 Rv3588 – F70, A75, Y89, V93 Nce103 – F97, L102, G111, D59	
Gate engineering		
PDB: ***WT (HICA)*** **–** 2A8D**,** ***WT (PPCA)*** **–** 1DDZ**,** ***WT (Rv3588)*** **–** 1YM3**,** ***WT (Nce103)*** **–** 3EYX		Tunnel
		Length
		Bottleneck
References: [93,138]		Role: Acetate ion, CO_2 transport

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Table 3. continued

14	Carboxylesterase pnbCE	3.1.1

Gate function
GATE1 – Controls exit of hydrolysis products by a side door
GATE2 – Controls access to the active site gorge
GATE3 – Controls access to the active site gorge

Enzyme function
Detoxification of xenobiotics

Gate location
GATE1 – Between active site and side door
GATE2 – Entrance to active site gorge
GATE3 – Entrance to active site gorge

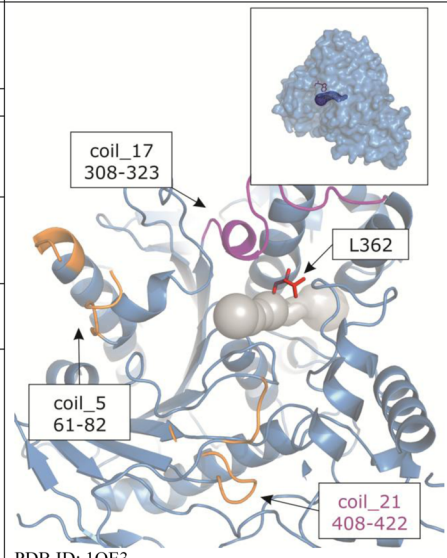
Gate structural basis

GATE1
L362
Rotates 180° around its C-C bond and adopts two distinct conformations

GATE2
Loop coil_5 (residues 61-82) and coil _21 (residues 408-422)
Move away from their equilibration conformation, the active site opens and can accommodate incoming substrate

GATE3
Loop coil_17(residues 308-323) and coil _21
Close for substrate hydrolysis, move away to release products

Gate engineering
Δ coil_5 – k_{cat} 4.5-fold smaller
Δ coil_21– k_{cat} 3-fold smaller



PDB ID: 1QE3

Tunnel		
Length		
Bottleneck		
Role		

PDB: *WT* – 1QE3

References: [139,140]

15	Catalase CAT-1 and CAT-3	1.11.1.6

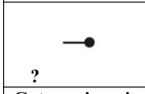
Gate function
GATE1 – Blocks access to the heme, controls solvation
?GATE2 – ?

Enzyme function
Decomposition of hydrogen peroxide to water and O_2

Gate location
GATE1 – Entrance to the active site cavity from main tunnel
?GATE2 – Side tunnel

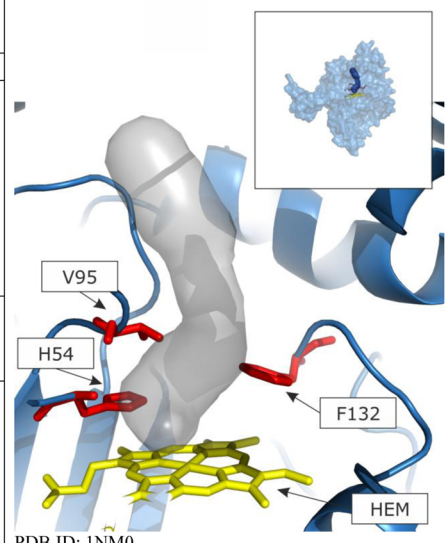
Gate structural basis

GATE1
H54, V95, and F132
Barrier to reach the active site:
H_2O_2 < 4.8 kcal/mol
O_2 ~2.2 kcal/mol
H_2O ~ 3.6 kcal/mol
Opening of the gate may be regulated according to the H_2O_2 concentration in the small cavity before the gate; H_2O_2 in the cavity would change the net of hydrogen bonds and trigger opening of the gate; water molecules interacting with amino acid residues in the cavity would determine closure of the gate



?GATE2 (not shown on picture)
CAT-1 – hydroxyl group of S198 blocks tunnel
CAT-3 – hydroxyl group of T208 blocks tunnel

Gate engineering



PDB ID: 1NM0

Tunnel	Main	Back
Length	31	
Bottleneck		
Role	O_2 and H_2O_2 transport	

PDB: *WT* – 1NM0

References: [23,24,88]

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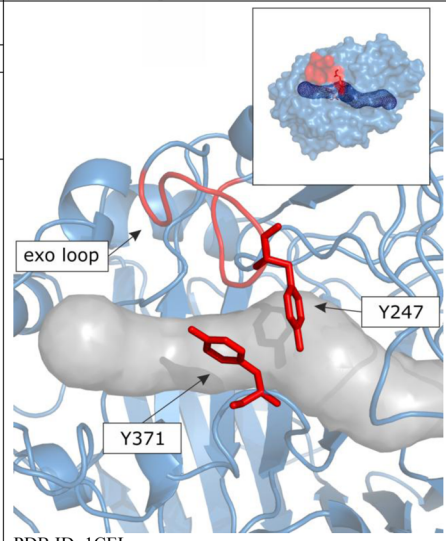
Table 3. continued

16	**Cellobiohydrolase CEL7A**	3.2.1.91
Gate function **GATE1** – Facilitates processing of crystalline cellulose degradation		**Enzyme function** Hydrolysis of amorphous and crystalline cellulose
Gate location **GATE1** – Exo loop		 PDB ID: 1CEL
Gate structural basis		Tunnel
	**GATE1** Exo loop + Y247 Y247 in closed conformations interacts with Y 371 from the short loop – acting like a button – it creates tunnel, loop covers the active site during reaction time	Length: 50
Gate engineering Y247F – removes hydrogen bond between Y247 and substrate - small effect on cellulose hydrolysis D241C/D249C – reduces mobility of the loop, disulphide bridge enhanced the activity on both amorphous and crystalline cellulose Deletion G245-Y252 – increases activity on amorphous cellulose, and half of the activity on crystalline cellulose		Bottleneck: 3.5
PDB: ***WT*** – 1CEL		Role: Substrate cavity
References: [49,141–143]		

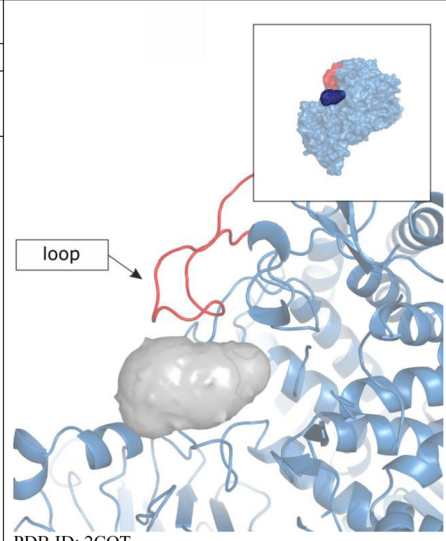
17	**Cellobiose Phosphorylase**	2.4.1.20
Gate function **GATE1** – Controls access of the substrate		**Enzyme function** Phosphorolysis of cellobiose into α-D-glucose 1-phosphate (G1P) and D-glucose
Gate location **GATE1** – Flexible loop		 PDB ID: 2CQT
Gate structural basis		Tunnel
	**GATE1** Flexible loop (495–513) Loop undergoes conformational changes during substrate binding and release	Length
Gate engineering T508I – changes substrate specificity N156D and N163D – increase of the activity N156D/N163D/T508I/E649G/N667A – improves activity towards a whole range of β-glucosidic acceptors		Bottleneck
PDB: ***WT*** – 2CQT, 1V7X		Role
References: [144,145]		

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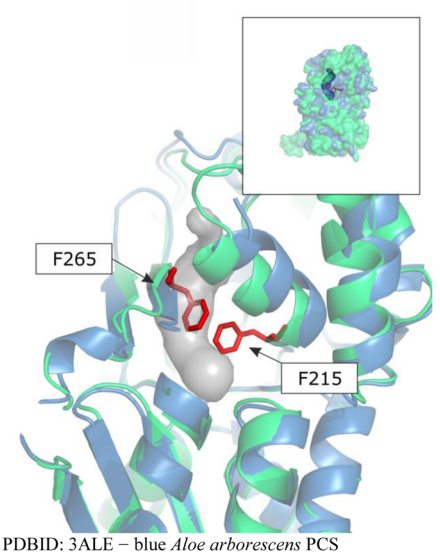
Table 3. continued

18	**Chalcone Synthase CHS**	2.3.1.74
Gate function GATE1 – Controls orientation of the substrate		**Enzyme function** Decarboxylative condensations of malonyl-CoA with a CoA-linked starter
Gate location GATE1 – Between the active-site cavity and the CoA binding tunnel		 PDBID: 3ALE – blue *Aloe arborescens* PCS PDBID: 1CGK – light green *Medicago sativa* CHS tunnel calculated for 3ALE structure
Gate structural basis		Tunnel
	GATE1 *Medicago sativa* CHS F215 and F265 Block the lower portion of the opening between cavities and help with folding and the internal orientation of the tetraketide intermediate during the cyclization reaction; F215facilitate the decarboxylation of malonyl-CoA by maintaining the orientations of substrates and intermediates during the sequential condensation reactions	Length: 16
Gate engineering *M. sativa* CHS F215S – changes the substrate specificity via opening a space at the cavity entrance *A. arborescens* PCS *GATE2 M207G – opens a gate to two novel hidden pockets behind the active site of the enzyme - residue 207 controls the number of condensations of malonyl-CoA F80A/Y82A/M207G – provide further elongation of products		Bottleneck:
PDB: ***WT (M. sativa CHS)*** **–** 1CGK, ***WT (A. arborescens PCS)*** **–** 3ALE		Role: Reaction cavity
References: [95,106,146]		

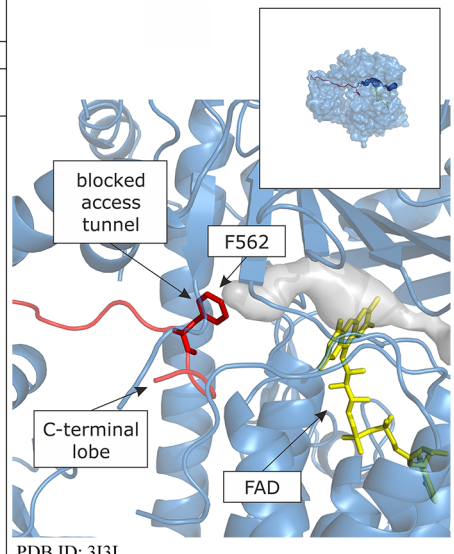
19	**Chloramphenicol Halogenase CmlS**	Not determined
Gatefunction GATE1 – Blocks access to the active site		**Enzyme function** Formation of the dichloroacetyl group
Gate location GATE1 – Flexible loop (the C-terminal lobe of CmlS)		 PDB ID: 3I3L
Gate structural basis		Tunnel
	GATE1 Flexible loop (the C-terminal lobe of CmlS) + F562 acting as a plug	Length: 10
Gate engineering		Bottleneck:
PDB: ***WT*** **–** 3I3L		Role: Connecting halogenation active site and FAD binding site
References: [147]		

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Table 3. continued

20	Cholesterol Oxidase – type 1 SCHOX	1.1.3.6

Gate function
GATE1 – Controls access of the solvent
GATE2 – Controls access of O_2 to the flavin and assures that isomerization occurs before the oxidative half of the reaction, tuning the redox state of the cofactor

Enzyme function
Catalyses the first step in the pathway of cholesterol degradation

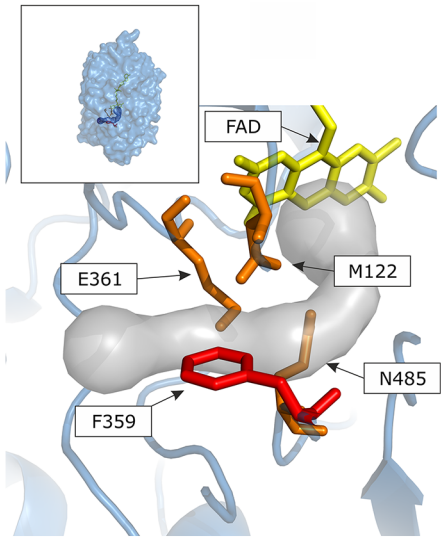
Gate location
GATE1 – Frames the entrance to the tunnel
GATE2 – Gate is in the tunnel leading to the isoalloxazine ring of flavin

Gate structural basis

GATE1
F359
F359 adopts two distinct alternate conformations separated by a 65° rotation of the benzene group
Closed gate - maximizing hydrophobic packing interactions with V189, V124 and G347
Open conformation - the tunnel becomes solvent accessible
Control of F359 gate appears to be dependent on the adopted conformation of N485

GATE2
N485, E361 and M122
Switch between their side-chain conformations, controls the access of O_2 to flavin
1. Binding of steroid → rotation of the methyl group of M122 → pushes N485 sealing the tunnel and creating an ideal environment for oxidation
2. After substrate oxidation, a strong hydrogen bond forms when N485 moves to conformation near the flavin, forcing M122 to a conformation that destabilizes the binding of the oxidized product.
3. When the substrate is oxidized and the FAD cofactor is reduced, the side chain of N485 rotates toward the cofactor → tunnel opening - regulates access of oxygen to the active site

Gate engineering
F359W – rate of catalysed reaction decreases 13-fold
G347N – could not be saturated with oxygen
N485D – could not be saturated with oxygen



PDB ID: 1MXT

Tunnel	
Length	32
Bottleneck	1.2
Role	O_2 and H_2O_2 transport

PDB: ***WT*** – 1MXT, ***Mutant F359W*** – 3CNJ, ***Mutant N485D*** – 3GYI

References: [57,99,148]

21	Cholesterol Oxidase – type II BsChOx	1.1.3.6

Gate function
GATE1 – Modulates access/reactivity of dioxygen

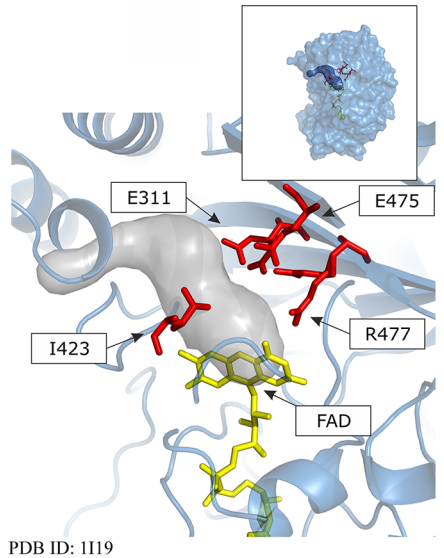
Enzyme function
Catalyses the first step in the pathway of cholesterol degradation

Gate location
GATE1 – Bottleneck of the tunnel

Gate structural basis

GATE1
E311, I423, E475 and R477
Open conformation of R477 is stabilized by a salt bridge with E311
E311 tunes the E475—R477 pair

Gate engineering
E311D/Q/L – cause a switch in the basic kinetic mechanism of the reoxidation with dioxygen, while BsChOx wild type and most mutants show saturation behaviour with increasing O_2
For E311 – a linear dependence was found that would reflect a second-order process
R477A – limits both oxidation and isomerization activities
A204C, G309A, G309C, I423L, I423V, E475D, E475Q, R477K – no significant changes



PDB ID: 1I19

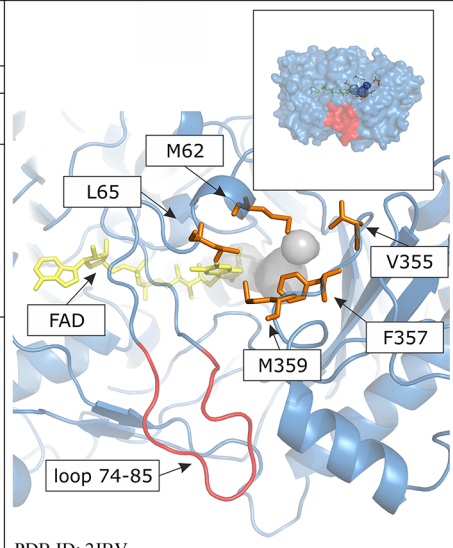
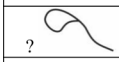
Tunnel	
Length	23
Bottleneck	1.4
Role	Oxygen transport

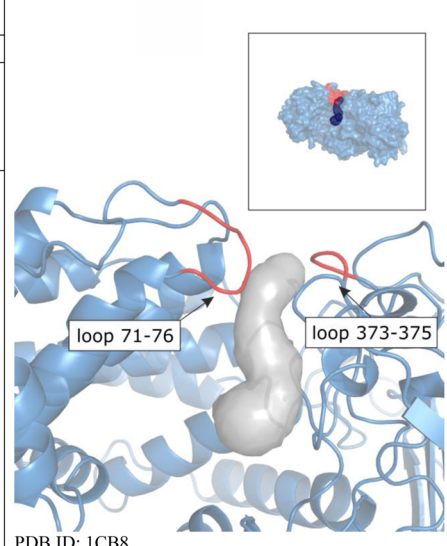
PDB: ***WT*** – 1I19

References: [99,149,150]

dx.doi.org/10.1021/cr300384w | *Chem. Rev.* 2013, 113, 5871−5923

Table 3. continued

22	Choline Oxidase	1.1.3.17
Gate function ?GATE1 – Controls access of the positively charged substrate GATE2 – Controls access of the positively charged substrate		**Enzyme function** Oxidation of choline to glycine betaine (N,N,N-trimethylglycine)
Gate location ?GATE1 – Loop adjacent to the active site of choline oxidase - residues 74-85 GATE2 – Located in the tunnel above active site		
Gate structural basis		
	?GATE1 Movement of the loop can open active site	
	GATE2 M62, L65, V355, F357, and M359 Breathing motion of M62, L65, V355, F357, and M359 The weakly hydrophobic interactions between the gating residues ensure that the positively charged substrate can easily slip to the highly electronegative active site The distribution of the residues just outside the five gating residues contains a considerable amount of negatively charged amino acids, which include E63, E66, E358, and E370, which may attract and guide the positively charged choline substrate to the active site	PDB ID: 2JBV
Gate engineering		Tunnel
		Length
PDB: ***WT*** **–** 2JBV		Bottleneck: 2.5
References: [31]		Role: Substrate transport

23	Chondroitin AC Lyase	4.2.2.5
Gate function GATE1 – Creates tunnel and the active site, controls access of the solvent		**Enzyme function** Degradation of glycosaminoglycans
Gate location GATE1 – N-terminal domain and C-terminal domain		
Gate structural basis		
	GATE1 Two loops Loops are flexible and open periodically allowing the glycosaminoglycan chain to slide in; Gating involves the movement of the tips of one or two loops only, D71-W76 of the N-terminal domain and G373-K375 of the C-terminal domain	PDB ID: 1CB8
Gate engineering		Tunnel
		Length
PDB: ***WT*** **–** 1CB8, 1HM2, 1HMU, 1HM3, 1HMW		Bottleneck
References: [151]		Role: Active site cavity

dx.doi.org/10.1021/cr300384w | *Chem. Rev.* 2013, 113, 5871−5923

Table 3. continued

24	Copper – Containing Amine Oxidase	1.4.3.21

Gate function
GATE1 – Blocks the back side of the active site from solvent access

Enzyme function
Oxidation of primary amines to aldehydes reducing molecular oxygen to hydrogen peroxide

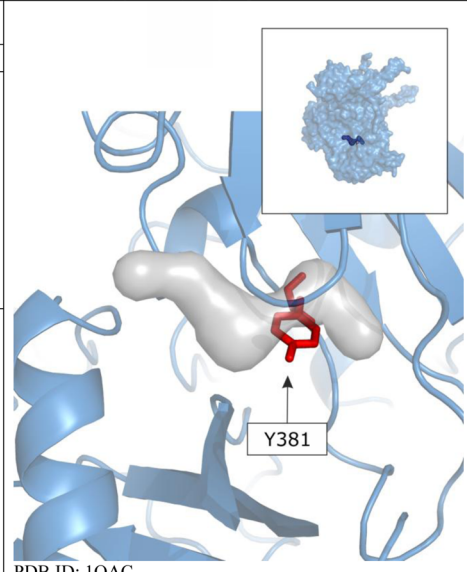
Gate location
GATE1 – Amine tunnel

Gate structural basis

GATE1
Y381 in amine oxidase ECAO
W156 in amine oxidase HCAO
Y296 in amine oxidase AGAO
F298 in amine oxidase PSAO

Gating residues can form a π/π ring stacking interaction with the pyridine ring of the cofactor (TPQ)

Gate engineering



PDB ID: 1OAC

Tunnel	
Length	
Bottleneck	
Role	

PDB: ***WT (ECAO)*** **–** 1OAC, ***WT (HCAO)*** **–** 2OQE, 2OOV, ***WT (AGAO)*** – 1RJO, 1RKY, ***WT (PSAO)*** **–** 1W2Z

References: [88,152–154]

25	Cytidine Triphosphate Synthetase CTPS	6.3.4.2

Gate function
GATE1 – Creates tunnel, protects tunnel from access of the solvent
?GATE2 – Controls passage of ammonia to the amidotransferase active site
GATE3 – Controls passage of ammonia to the amidotransferase active site

Enzyme function
Catalyses the formation of CTP from UTP, ATP and glutamine

Gate location
GATE1 – Loop of the glutaminase domain
?GATE2 – The domain interface, interacts with the amide group of the bound glutamine
GATE3 – Between UTP site and the tunnel exit

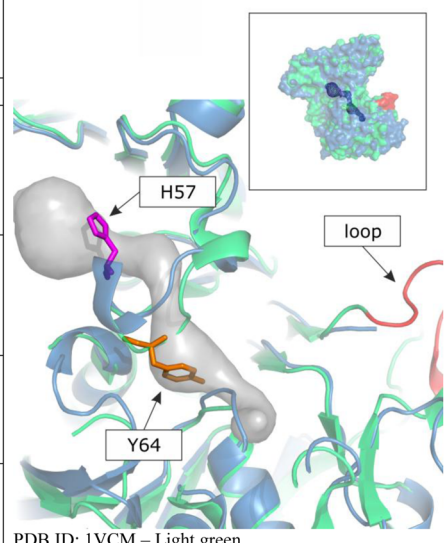
Gate structural basis

GATE1(PDB ID: 1VCM)
Loop
The synthetase active site is exposed to the solvent and the binding pocket for the allosteric effector GTP is not properly formed
After ATP and acceptor binding loop covers the entrance of the glutaminase site shielding glutamine and creates the tunnel

?GATE2 (PDB ID: 1VCM)
Y64
Y64 might act as a door to the ammonia tunnel leading to the synthetase site

GATE3 (PDB ID: 2ADP)
H57
1. Binding of the substrate UTP induces the rotation of H57
2. Rotation of H57 opens the tunnel for ammonia passage
The ligand-induced change is postulated to regulate the timing for the translocation of ammonia

Gate engineering



PDB ID: 1VCM – Light green
PDB ID: 2AD5 – Blue
Tunnel calculated for PDB ID: 2AD5 structure

Tunnel between active sites	
Length	25
Bottleneck	0.8-1.2
Role	NH_3 transport between active sites

PDB: ***WT*** **–** 1VCM, 1VCN, 1VCO, 2AD5, 1S1M

References: [52,155–157]

dx.doi.org/10.1021/cr300384w | *Chem. Rev.* 2013, 113, 5871–5923

Table 3. continued

26	**Cytochrome P450 CYP3A4**	1.14.13.32

Gate function
Controls access to the active site, controls selectivity and specificity of the enzyme

Enzyme function
Catalyses mono-oxygenation reactions such as hydroxylation and epoxidation, major drug metabolizing enzyme in humans

Gate location
Depends on the P450 isoform, tunnel and the gate

Gate structural basis

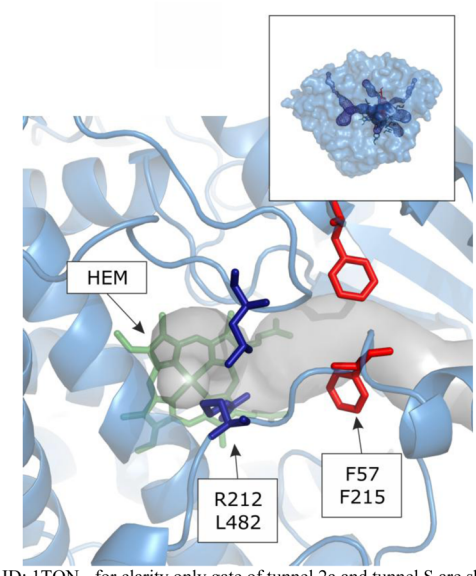
Common gating mechanisms in cytochrome P450 family are:
(i) associated with F-G-helix-loop-helix or B-C loop movement (tunnels 2a, 2ac, 2b, 2c, 2e);
(ii) associated with swinging door type;
(iii) associated with wing type;
(iv) cofactor assisted (CPR)
For details of other cytochrome P450 gates see references [16,59,90]

CYP3A4

π-stacking, aliphatic contact, H-bond, salt bridge, protein conformation changes

Tunnel	Proposed gating residues for product release	
	TMZ	TST-OH
2a	**F57-F215**	**F57-F215**
2b	F108-F220	F108-F220
2c	F108-F241	F108-F241
2e	F108-I120	R105-S119
3	F213-F241	F213-V240
S	**R212-L482**	**R212-L482**
W	R375 + CPR protein	

Mechanism of gates opening:
(i) breaking F-F π-stacking
(ii) breaking aliphatic contacts between R212 and L482 - S tunnel
(iii) breaking the H bond, as observed during the exit of TST-OH through tunnel 2e between R105 and S119
(iv) opening of water tunnel when the FMN domain of CPR binds to CYP3A4 via R375 rotation.



PDB ID: 1TQN - for clarity only gate of tunnel 2a and tunnel S are shown

Gate engineering
F108W, I120W – closure of tunnel 2e, switching of the midazolam entrance to another tunnel that may lead to a different active site orientation and product formation

Tunnel	2a	2b	2c	2e	3	S	W
Length	19	18	17	12	17	13	
Bottleneck	4.1	4.0	3.9	4.2	4.1	4.5	
Role	Different products pathways					Proton access/ water/substrate	Water

PDB: ***WT*** **–** 1TQN (CYP3A4), 1AKD (P450cam), 2D09 (CYP152A2), 1JPZ (P450-BM3), 1PQ2 (CYP2C8), 1IZO (CYP152A1), 1F4T (CYP119), 1NR6 (CYP2C5), 1OXA (P450eryF)

References: [10,16,25,55,56,58,59,90,158,159]

27	**Dihydrofolatereductase**	1.5.1.3

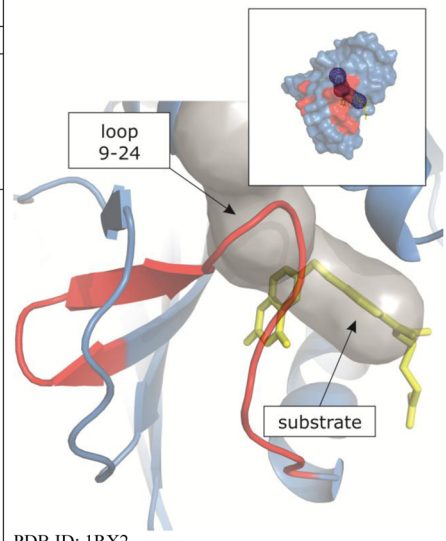
Gate function
GATE1 – Controls the activity

Enzyme function
Catalyses the stereospecific reduction of dihydrofolate to tetrahydrofolate

Gate location
GATE1 – Entrance to the cofactor cavity

Gate structural basis

GATE1
M20 loop (residues 9 to 24)
In closed conformation the loop packs tightly against the nicotinamide ring of the cofactor
In occluded conformation the loop projects into the active site and sterically blocks the cofactor cavity



PDB ID: 1RX2

Gate engineering
N23PP – k_{cat} decreased 5-fold
S148A – k_{cat} decreased 2-fold
N23PP/S148A – k_{cat} decreased 6-fold

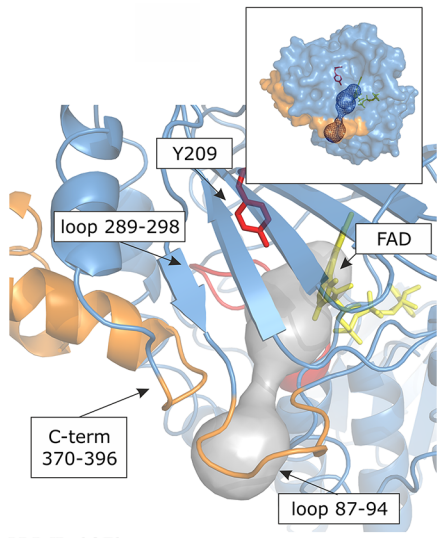
Tunnel	
Length	
Bottleneck	
Role	

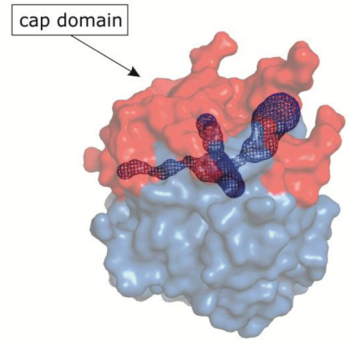
PDB: ***WT*** **–** 1RX2, **Mutants** – 3QL0, 3QL3

References: [80,81,160–162]

dx.doi.org/10.1021/cr300384w | *Chem. Rev.* 2013, 113, 5871–5923

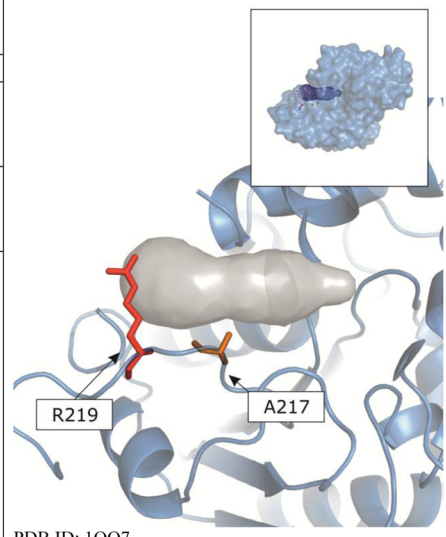
Table 3. continued

28	**Digeranylgeranylglycerophospholipid Reductase DGGR**	1.3.1
Gate function **GATE1** – Opens tunnel, controls entry of the substrate, controls reduction of FAD **GATE2** – Controls substrate binding/release		**Enzyme function** Converts 2,3-di-O-geranylgeranylglyceryl phosphate to 2,3-di-O-phytanylglyceryl phosphate (archaetidic acid)
Gate location **GATE1** – Vicinity of the cofactor binding site (FAD) **GATE2** – C-terminal helical subdomain		
Gate structural basis		
	**GATE1** Glycine-rich αI–αJ loop (residues 289–298) Cofactor FAD has two conformation IN (not accessible by solvent) and OUT (exposes the soalloxazine ring allowing it to be reduced by NADH (or NADPH). In IN conformation tunnel A is blocked, to open it has to turn into OUT conformation. The conformational changes in the glycine-rich αI–αJ loop disrupt interaction between Y209 and T292 allowing change of the FAD position. Conserved RxxFD and LxGD motifs may play a role in FAD's IN/OUT conformational switch.	
	**GATE2** Two regions, the β6–β7 loop (residues 87–94) and the C-terminal helices (residues 370–396) The conformation changes of the C-terminal helical subdomain may be involved in substrate binding/release	
Gate engineering		PDB ID: 3OZ2
		Tunnel / A / B
		Length
PDB: ***WT*** **–** 3OZ2		Bottleneck
References: [100]		Role

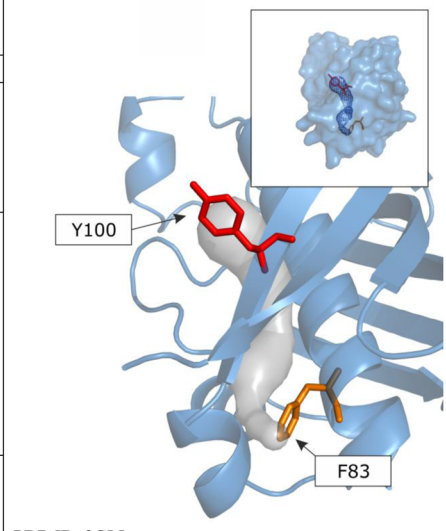
29	**Epoxide Hydrolase H37Rv**	3.3.2.3
Gate function **GATE1** – Regulates access to the active site		**Enzyme function** Hydrolysis of epoxides
Gate location **GATE1** – Cap domain		
Gate structural basis		
	**GATE1** (*Mycobacterium tuberculosis* H37Rv) Movement of the cap domain regulates access to the active site	
Gate engineering		PDB ID: 2E3J
		Tunnel
		Length
PDB: ***WT (Aspergillus niger M200)*** – 1QO7		Bottleneck
References: [68]		Role

dx.doi.org/10.1021/cr300384w | *Chem. Rev.* 2013, 113, 5871−5923

Table 3. continued

30	**Epoxide Hydrolase M200**	3.3.2.3
Gate function ?**GATE1** – Controls enantioselectivity and activity ?**GATE2** – Controls enantioselectivity and activity		**Enzyme function** Hydrolysis of epoxides
Gate location ?**GATE1** – Tunnel entrance ?**GATE2** – Middle of the tunnel		 PDB ID: 1QO7
Gate structural basis		
?	?**GATE1** (*Aspergillus niger M200*) R219	
?	?**GATE2** (*Aspergillus niger M200*) A217	
Gate engineering *Aspergillus niger M200* A217C, A217E, A217G, A217L, A217P, A217Q, A217R, A217T, A217V – at the entrance to the tunnel result in different enantioselectivity and activity; e.g.: A217G mutation results in a 33-fold decrease of activity A217V 6,6-fold increase of activity with no changes in the products enantioselectivity for the reaction with allyl glycidyl		
PDB: ***WT (Aspergillus niger* M200)** – 1QO7		
References: [26,163]		

Tunnel	C	N terminal cleft (not shown)
Length	25	15
Bottleneck		
Role	Substrate binding cavity	sEH N terminal cleft

31	**FabZ β-Hydroxyacyl-Acyl Carrier Protein Dehydratase**	4.2.1.59
Gate function **GATE1** – Controls access of the substrate, prohibits binding of small hydrophobic molecules to the unliganded enzyme **GATE2** – Controls length of the substrate		**Enzyme function** Elongation cycles of both saturated and unsaturated fatty acids biosyntheses in the type II fatty acid biosynthesis system (FAS II) pathway
Gate location **GATE1** – Entrance to the tunnel **GATE2** – Exit (back door) of the tunnel		 PDB ID: 2GLL
Gate structural basis		
	GATE1 Y100 (Y88 in PaFabZ and L170 in PfFabZ) adopts either an open or closed conformation in the crystal structure In closed conformation - Y100 is stabilized by the Van der Waals interactions with M102, M154, and P112; in open one it flops ~120° and is stabilized by the Van der Waals interactions with M154, K62, and I64	
	GATE2 F83 Refined in two additional alternative conformations, leading the tunnel to form a L-shape or an U-shape, in closed conformation - F83 points toward I98, in open position it rotates ~120° points toward I93, exposing the exit to the bulk solvent T85 or F74 play a similar role in PaFabZ	
Gate engineering Y100A – drops the activity of the mutant to less than 50% of the enzymatic activity of the wild type, the new entrance has ~15 Å in width, completely exposes the active site to the bulk solvent – as a results ACP binds to the mutant more stronger than to the wild-type. In particular, the dissociation step of ACP from the HpFabZ mutant is extremely slow.		
PDB: ***WT (HpFabZ)*** – 2GLL, 2GLP, 2GLM ***Mutant Y100A*** – 2GLV, ***WT (PfFabZ)*** – 1ZHG, ***WT (PcFabZ)*** – 1U1Z		
References: [54,164,165]		

Tunnel		
Length	20	
Bottleneck		
Role	Substrate binding tunnel, ACP binding groove	

dx.doi.org/10.1021/cr300384w | *Chem. Rev.* 2013, 113, 5871−5923

Table 3. continued

32	Formiminotransferase-Cyclodeaminase FTCD	2.1.2.5

Gate function
GATE1 – Controls position of H82 in proper orientation for reaction

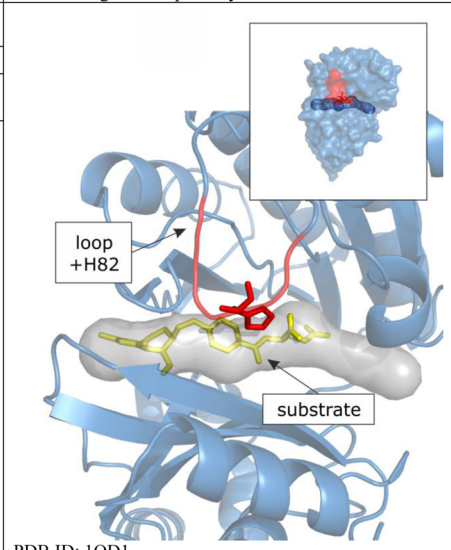
Enzyme function
Two independent, but sequential reactions in the histidine degradation pathway

Gate location
GATE1 – Blocks entrance from a short tunnel into active site

Gate structural basis

GATE1
Loop rearrangement including a change in the histidine H82 side-chain position

Gate engineering



PDB ID: 1QD1

PDB: ***WT*** – 1QD1

References: [94]

Tunnel	Main	Second
Length	38	9
Bottleneck	8	4
Role	Intermediate transport	Formiminoglutamate substrate entry, glutamate product exit

33	Glucosamine 6 Phosphate Synthase GlmS	2.6.1.16

Gatefunction
GATE1 – Protects Fru 6P site from the solvent, creates the ammonia tunnel
GATE2 – Protects glutaminase active site from the solvent
GATE3 – Opens ammonia tunnel, ammonia enters the sugar site, acts as a solvent barrier
GATE4 – Opens ammonia tunnel

Enzyme function
Catalyses glucosamine-6P synthesis from fructose-6P and glutamine

Gate location
GATE1 – Part of the active site cavity
GATE2 – Part of the active site cavity
GATE3 – Tunnel between two active sites
GATE4 – Bottleneck of the tunnel between active sites

Gate structural basis

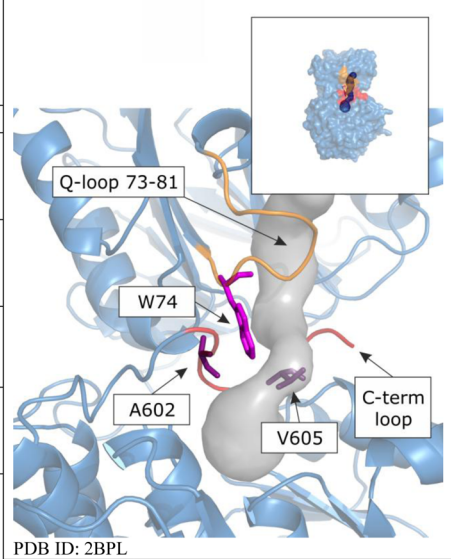
GATE1
C-terminal loop (residues 600–608)
Open state – relaxed loop
Closed state – anchored by Y28 and W74 with Q loop

GATE2
Q-loop (residues 73–81)
Open state - anchored by R539*,
Close state - after Q enters the active site anchored by Y28 and W74 with C-terminal loop

GATE3
W74
x1 torsion angle of W74 changes by 75°

GATE4
A602 and V605
A shift of the side-chain of the A602 residue is concomitant to a re-orientation of its backbone carbonyl group, which can then form a strong H-bond with the hydroxyl group of the Y28

Gate engineering
W74A, W74L, W74F – inefficient ammonia transfer
A602L, V605L – efficiency of ammonia transfer decreased 2-fold



PDB ID: 2BPL

PDB: ***WT*** – 2J6H, 1JXA, 2VF4, 2BPL

References: [14,45,50,155,166,167]

Tunnel	
Length	18
Bottleneck	
Role	Ammonia transport

dx.doi.org/10.1021/cr300384w | *Chem. Rev.* 2013, 113, 5871–5923

Table 3. continued

34	**Glutamate Synthases GltS**	1.4.7.1

Gate function
GATE1 – Controls correct conformation of active site for L-glutamine binding and hydrolysis, crucial for glutaminase activation and coupling of the glutaminase and synthase sites, creates ammonia tunnel
GATE2 – Shields substrate from bulk solvent
GATE3 & **GATE4** – Controls transport of ammonia

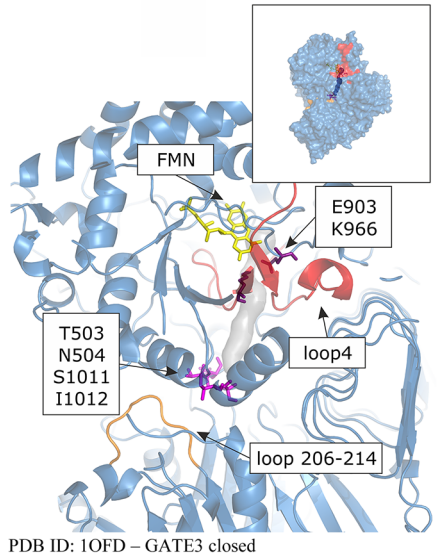
Enzyme function
Formation of 2 L-glutamate from L-glutamine and 2-oxoglutarate

Gate location
GATE1 – Loop protecting Fru 6P site from solvent
GATE2 – Loop protecting glutaminase site from solvent
GATE3 – Entrance to the interdomain tunnel
GATE4 – Entrance to the synthase site (end of the tunnel)

Gate structural basis

	**GATE1** Loop 4 (residues 933–978) Shifts to the active conformation – C-terminal residue E1013 forms a hydrogen bond with C1 and keeps correct conformation of C1 and loop 29–34 for L-glutamine binding and hydrolysis. E1013 side chain may also play a role in the precise geometry of the tunnel entry point
	**GATE2** Loop 206–214 Closing of loop 206–214 after L-glutamine binding
	GATE3 T503, N504, S1011 and I1012 Small conformation changes of T503, N504 of the central domain, S1011 and I1012 of loop 4
	GATE4 E903 and K966 Small conformation changes of E903 and K966 Additionally residues T507 and N508 and S976 and I977 may function as a gates for signalling between active sites

Gate engineering
E1013D – 100-fold decrease of activity, a sigmoid dependence of initial velocity on L-glutamine concentration
E1013N – 1000-fold decrease of activity, exhibited hyperbolic kinetics
E1013A – 1000-fold decrease of activity



PDB ID: 1OFD – GATE3 closed

Tunnel	
Length	33
Bottleneck	
Role	NH_3 transport, prevents protonation of NH_3

PDB: ***WT*** – 1OFD, 1OFE, 1EA0

References: [46,62,155,168]

35	**Glutamine Phosphoribosylpyrophosphate Amidotransferase**	2.4.2.14

Gate function
GATE1 – Closes synthase site and creates a narrow, solvent-inaccessible tunnel between active sites
GATE2 – Covers glutaminase site
GATE3 – Protects tunnel from the solvent access and avoids wasteful release of ammonia into solution

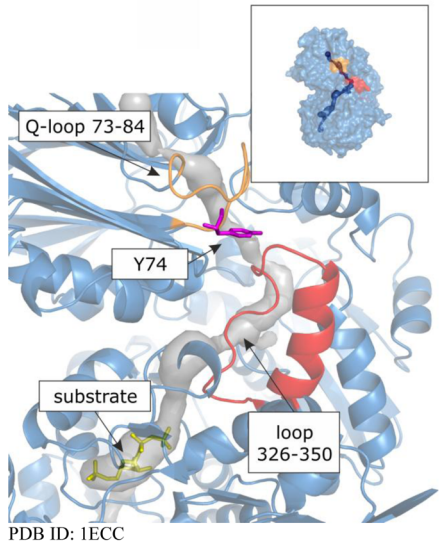
Enzyme function
Catalyses the initial reaction in *de novo* purine nucleotide biosynthesis

Gate location
GATE1 – Loop near the synthase active site
GATE2 – Loop near the glutaminase active site
GATE3 – On Q-loop

Gate structural basis

	**GATE1** Loop (residues 326–350) The active site cavity is created by closure of the loop (residues 326–350) Closing of the active site creates a narrow, solvent-inaccessible tunnel between active sites I335 interacts with Y74
	**GATE2** Q-loop (residues 73–84)
	GATE3 Y74 A key residue in coupling the glutamine and acceptor sites upon acceptor binding may play a similar gate role as W74 in GlmS

Gate engineering



PDB ID: 1ECC

Tunnel between two active sites	
Length	20
Bottleneck	
Role	Ammonia transport

PDB: ***WT*** – 1ECF, 1ECC

References: [155,169,170]

dx.doi.org/10.1021/cr300384w | *Chem. Rev.* 2013, 113, 5871−5923

Table 3. continued

36	Haloalkane Dehalogenase DhaA	3.8.1.5

Gate function
GATE1, **GATE2**, **GATE3**, **GATE4**, **GATE5** – Controls access to the tunnel

Gate location
GATE1 –Tunnel p1,**GATE2** –Tunnel p2a, **GATE3** –Tunnel p2b, **GATE4** –Tunnel p2c, **GATE5** –Tunnel p3

Gate structural basis

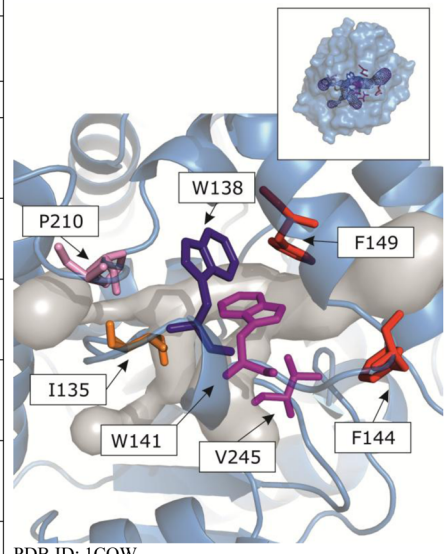
●— —●	**GATE1** F144 and F149
—●	**GATE2** I135
●— —●	**GATE3** V245, W141
●— —●	**GATE4** β-bridge interaction between P210 and A212 of the CC loop and I135 of the NC loop
—●	**GATE5** W138 blocks tunnel p3

Gate engineering
C176Y, V245F, A172F, A145F – limiting access of water to the active site

PDB: ***WT (DhaA)*** **–** 1CQW, 1BN6, 1BN7

References: [109,110]

Enzyme function
Hydrolytic dehalogenation of various halogenated aliphatic hydrocarbons



PDB ID: 1CQW

Tunnel	p1	p2a, p2b, p2c	p3
Length	20	22	24
Bottleneck	2.8	1.8	1.6
Role	Product	Product	Product

37	Haloalkane Dehalogenase LinB	3.8.1.5

Gate function
GATE1 – Controls access to the active site
GATE2 – Controls selectivity

Gate location
GATE1 – Tunnel bottleneck
GATE2 – Tunnel bottleneck

Gate structural basis

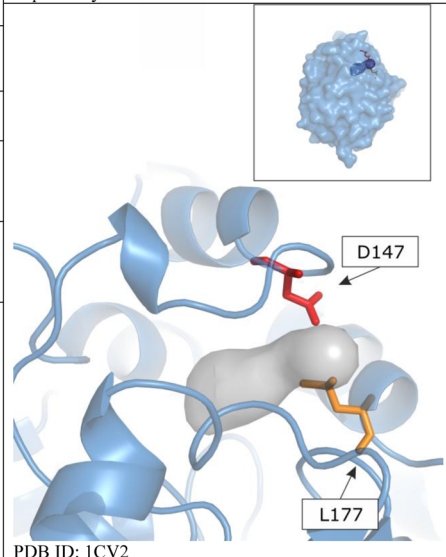
—●	**GATE1** D147 Motion of this residues is necessary for entry of large substrates
—●	**GATE2** L177 Partially blocks the entrance of the main tunnel

Gate engineering
L177A, L177C, L177G, L177F, L177K, L177T, L177W, L177D, L177H, L177M, L177Q, L177R, L177S, L177V, L177Y influence the substrate specificity and activity

PDB: ***WT (LinB)*** **–** 1CV2

References: [72,108,171]

Enzyme function
Hydrolytic dehalogenation of various halogenated aliphatic hydrocarbons



PDB ID: 1CV2

Tunnel	p1
Length	12
Bottleneck	2.6
Role	Product

dx.doi.org/10.1021/cr300384w | *Chem. Rev.* 2013, 113, 5871−5923

Table 3. continued

38	**Histone Deacetylase HDAC1 and HDAC2**	3.5.1.98

Gate function
GATE1 – Controls access to the active site through tunnel A
GATE2 – Controls access to the active site through tunnel B1
GATE3 – Controls access to the active site through tunnel B2 (only HDAC1)

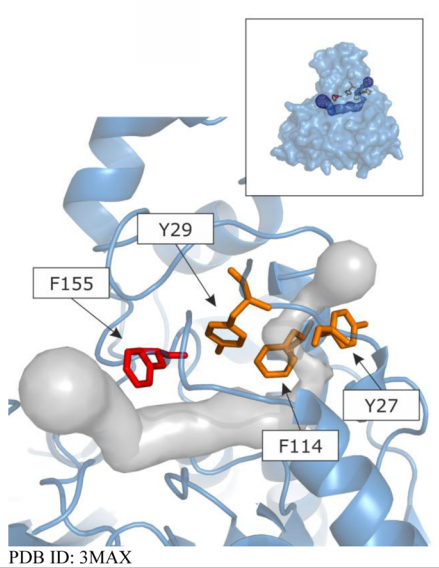
Enzyme function
Modifies chromatin structures by removing the acetyl group from the ε-amino lysine residues

Gate location
GATE1 – Bottleneck of tunnel A
GATE2 – Bottleneck of tunnel B1
GATE3 – Bottleneck of tunnel B2 (only HDAC1)

Gate structural basis

—●	**GATE1** F155 (F150) Side chain of F155 has to rotate ~ 180°
●— —●	**GATE2** F114 + Y27 and Y29 (F109 + Y22 and Y24) F114 residue is located between two tyrosine rings in closed conformation Opening requires unzipping of the gate
—●	**GATE3** Y303 Observed only in HDAC1 - Y303 residue may rotate opening access through tunnel – anchoring residue in closed position M30

Gate engineering



PDB ID: 3MAX

Tunnel	A	B1	B2
Length	11	14	16
Bottleneck			
Role	Access to the active site	Access to the active site	Water exchange

PDB: ***WT (HDAC2)*** **–** 3MAX

References: [172]

39	**Histone Deacetylase HDAC8**	3.5.1.98

Gate function
GATE1 – Regulates water or product (acetate) transit from the active site through the internal tunnel

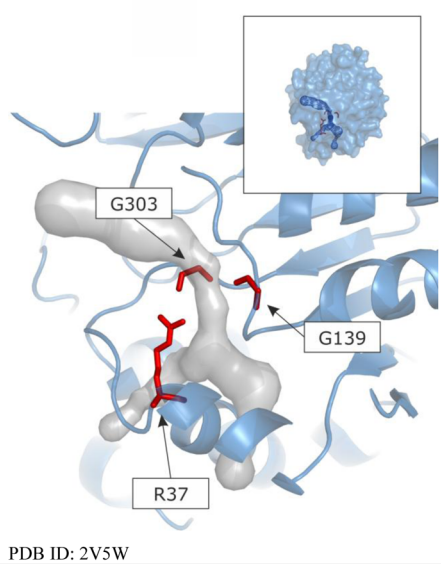
Enzyme function
Deacetylation of the Ɛ-amino group of specific lysine residues within histones and other proteins

Gate location
GATE1 – Centre of the internal tunnel

Gate structural basis

—●	**GATE1** R37 A structural reorientation of R37 and the loop is required for opening the access to the active site via the 14 Å 'internal' tunnel. R37 forms multiple hydrogen bond interactions with the backbone carbonyl oxygen atoms of conserved G303 and G305 positioned in a loop between the β8and α10-helix Gating interaction between G139 and G303

Gate engineering
R37A – the values for k_{cat}/K_M decrease 530-fold



PDB ID: 2V5W

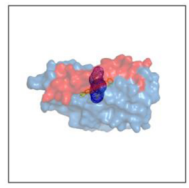
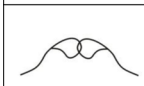
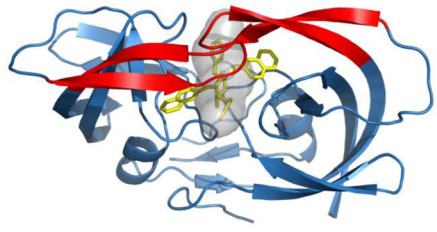
Tunnel		
Length	11	14
Bottleneck		
Role	Access to active site	

PDB: ***WT*** – 2V5W

References: [173]

dx.doi.org/10.1021/cr300384w | *Chem. Rev.* 2013, 113, 5871−5923

Table 3. continued

40	HIV-1 Protease	3.4.23.16
Gate function **GATE1** – Controls access to the active site		**Enzyme function** Central role in processing HIV-1 viral polypeptide precursors
Gate location **GATE1** – β-turn flaps		
Gate structural basis		
	**GATE1** β-turn flaps (residues 43–58) Movement of two β-turn flaps controls access to the active site	
Gate engineering WT – open 14% of time (Brownian dynamics simulations) G48V/V82A – open 2% of time (Brownian dynamics simulations) I84V/L90M – open 2% of time (Brownian dynamics simulations) L90M, G48V – open 14% of time (Brownian dynamics simulations) F53L – unstabilised semi open conformation due to lack of F53-I50 interaction		 PDB ID: 1HVR
		Tunnel
		Length:
PDB: ***WT*** **–** 1HHP, 1HVR		Bottleneck:
References: [79,89,174–177]		Role: Active site gorge

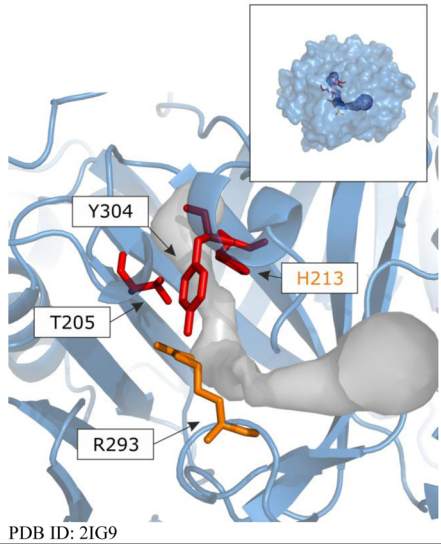
41	Homoprotocatechuate 2,3-Dioxygenase	1.13.11.15
Gate function **GATE1** – Controls O_2 diffusion pathway **GATE2** – Controls alternative O_2 diffusion pathway		**Enzyme function** Degradation of catechol and its derivatives by cleavage of aromatic rings
Gate location **GATE1** – Just below the protein surface **GATE2** – Below the protein surface		 PDB ID: 2IG9
Gate structural basis		
	**GATE1** T205, H213, and W304 Breathing motion of protein causes synchronizing movement of residues	
	GATE2 R293, H213 The fluctuation of R293 along with H213 could result in opening of the pathway	
Gate engineering		
		Tunnel
		Length:
PDB: ***WT*** **–** 2IGA, 2IG9		Bottleneck:
References: [63]		Role: O_2 transport

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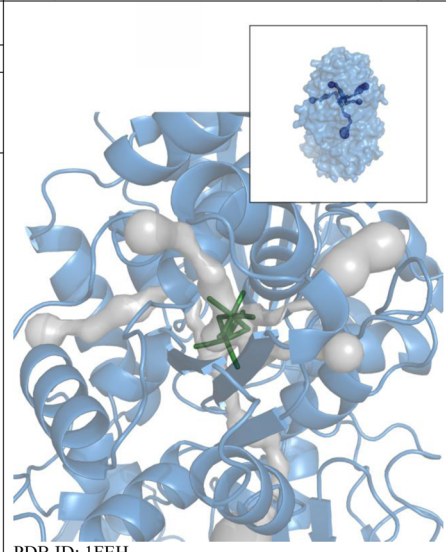
Table 3. continued

42	**Hydrogenase FeFe**	1.12.7.2
Gate function Controls access of oxygen and hydrogen to the active site		**Enzyme function** Catalyses the reversible oxidation of molecular hydrogen
Gate location Not specified, between dynamic cavities		 PDB ID: 1FEH Representative tunnels connecting internal cavities calculated with probe radius 0.6 Å
Gate structural basis		Tunnel
	No permanent tunnel – O_2 moves from cavity to cavity as the cavities fluctuate inside the protein	Length: 30 – 35
Gate engineering		Bottleneck: 2
PDB: ***WT*** **–** 1HFE, 1FEH		Role: H_2 and O_2 transport
References: [88,178–180]		

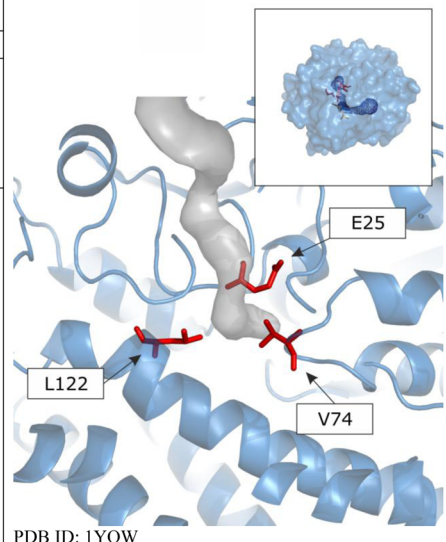
43	**Hydrogenase NiFe**	1.12.2.1
Gate function ?**GATE1** – Protects the active site against O_2		**Enzyme function** Catalyses the reversible oxidation of molecular hydrogen
Gate location ?**GATE1** – Vicinity of NiFe cluster, bottleneck close to the active site		 PDB ID: 1YQW
Gate structural basis		Tunnel
	?**GATE1** E25, V74 and L122 The size of the amino acids at positions 122 and/or 74 may determine the accessibility of the active site and therefore the resistance to O_2	Length: 30
Gate engineering V74Q, V74M, V74E, V74N, V74W, V74F, V74D, L122M/V74M, L122F/V74I, L122A/V74M – two contributions, size and polarity, are independent and have different effects to H_2, CO, O_2 molecules, different influences on reaction rate and inhibition effect		Bottleneck:
PDB: ***WT*** **–** 1YQW ***Mutants*** **–** 3CUR, 3CUS, 3H3X		Role: O_2 transport
References: [21,22]		

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Table 3. continued

44	**Imidazole Glycerol Phosphate Synthase IGPS**	2.4.2

Gate function
GATE1 – Prevents penetration of bulk water molecules into chambers I and II
GATE2 – Controls ammonia transfer, discriminates between ammonia and water

Enzyme function
Catalyses the closure of the imidazole ring within histidine biosynthesis and provides 5-aminoimidazole -4-carboxamide ribotide (AICAR) for use in the *de novo* synthesis of purines

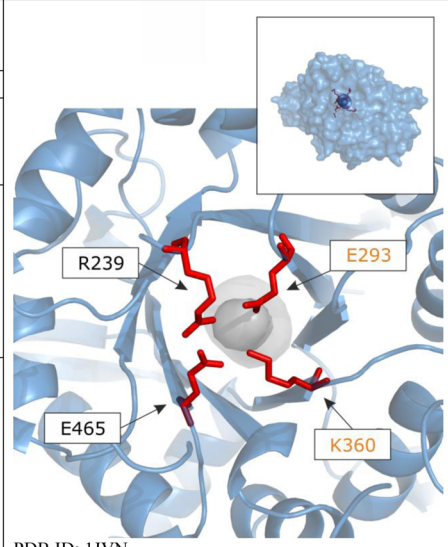
Gate location
GATE1 – Near the entrance of the $(\beta\alpha)_8$ barrel of hisF
GATE2 – Between chamber I and chamber II

Gate structural basis

GATE1 (for water)
R5 (R239), E46 (E293), K99 (K360), and E167(E465)
numbers from yeast or (*Thermotoga maritima*)
Four strictly conserved gate residues act as the wall barrier for water molecules

GATE2 (for ammonia)
K99 (K360) and E46 (E293)
Conformational change of all residues – high barrier 25 kcal/mol for ammonia transfer
K99 (K360) side chain rotation barrier 10 kcal/mol
Both K99 and R5 – fully open gate barrier 2 kcal/mol
Ammonia enters to chamber II through a side-opening between residues K99 and E46, the only requisite is the slight bending of the side chain of K99 (K360) - eliminates the need for an energetically costly gate-opening mechanism

Gate engineering
Y138F – experimentally no change of kinetics and stoichiometry, in simulations Y138 is not a gate but prevents bulk water from entering the interface during a reaction, keeping ammonia sequestered within the tunnel
R5A (R239A) – loss of ammonia through new hole and results in a 10^3 decrease in k_{cat}/K_M values for the cyclase reaction, in simulations possibly because the mutation allowed water molecules to access chamber II; these additional water molecules increased the energetic barrier to ammonia entry and passage through the tunnel
K99A (K360A) – experimentally 3-fold decrease in the overall reaction stoichiometry, in sumulations larger opening between chamber I and chamber II, and simultaneously deprives E167 and E46 of a salt-bridge partner
T78A – allowed a rapid and unhindered conduction of ammonia through the tunnel
T78F, P76F –block the ammonia conduction
Any mutation K181 (K196), D98 (D359), and Q123 (Q387) – decouples the two reactions



PDB ID: 1JVN
Gate in closed position blocking the tunnel

Tunnel	
Length	22
Bottleneck	3.8
Role	Ammonia transport

PDB: ***WT*** **–** 1KA9, 1JVN

References: [38,43,155,181–186]

45	**Inosine 5'- Monophosphate Dehydrogenase**	1.1.1.205

Gate function
GATE1 – Conformational change converts the enzyme from a dehydrogenase into hydrolase

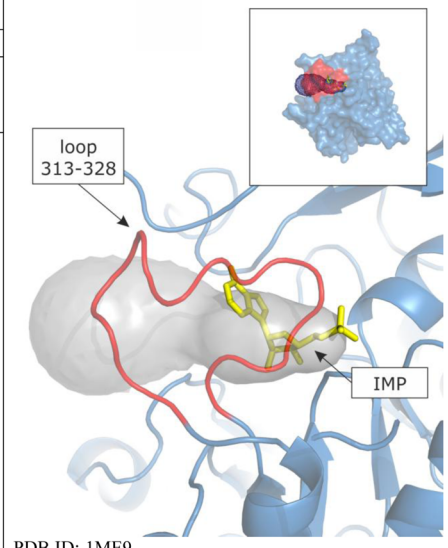
Enzyme function
Transformation of inosine 5'-monophosphate into xanthosine 5'-monophosphate

Gate location
GATE1 – Loop covering active site cavity

Gate structural basis

GATE1
C319 loop (313-328) adopts different conformations during the dehydrogenase and hydrolase reactions

Gate engineering



PDB ID: 1ME9

Tunnel	
Length	
Bottleneck	
Role	

PDB: ***WT*** **–** 1ME9, 1LRT, 1ME8, 1PVN

References: [187]

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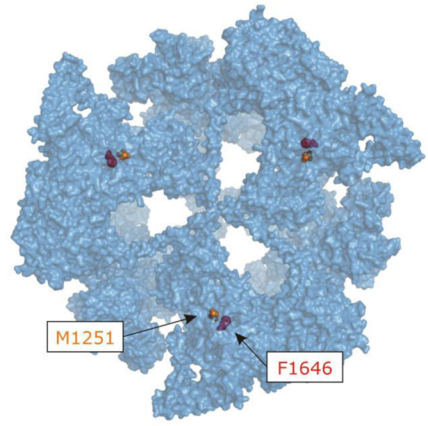
Table 3. continued

46	**Ketoacyl Synthase KS**	2.3.1.86
Gate function GATE1 – Shields the active site GATE2 – Controls access of the substrate		**Enzyme function** Elongates an ACP- or CoA-associated acyl chain by adding C2 units through a ping-pong decarboxylating condensation mechanism
Gate location GATE1 – Entrance to the active site GATE2 – Centre of the acyl-binding tunnel		
Gate structural basis		PDB ID: 2VKZ
—●	GATE1 F1646 Shields the active site, flips and allows access to the nucleophilic cysteine	
—●	GATE2 M1251 rotates and unlocks the inner part of the fatty acid binding cavity	
Gate engineering		
PDB: ***WT*** **–** 2VKZ		Tunnel Length Bottleneck Role
References:[188]		

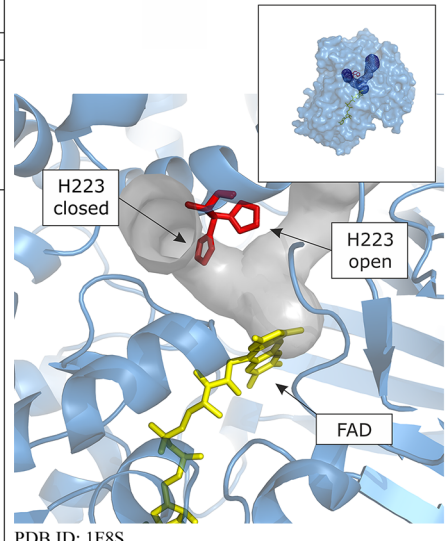
47	**L-Amino Acid Oxidase**	1.4.3.2
Gate function GATE1 – Controls access to the oxygen tunnel, binds the substrate initially		**Enzyme function** Oxidative deamination of L-amino acid substrates
Gate location GATE1 – 15 Å from surface in main funnel		
Gate structural basis		PDB ID: 1F8S
—●	GATE1 H223 H223 has two conformations and can act as a gate and binds the substrate initially H223 conformation A closing oxygen tunnel → substrate entry and deprotonation of the zwitterion → substrate into Michaelis position →H223 turn into conformation B → opening of oxygen tunnel	
Gate engineering		
PDB: ***WT*** **–** 1F8R, 1F8S		Tunnel: Main funnel / Y shape tunnel Length: 25 / 9 Bottleneck: 4.2 / 2 Role: Access to active site / O_2 access and H_2O_2 release
References: [73,189]		

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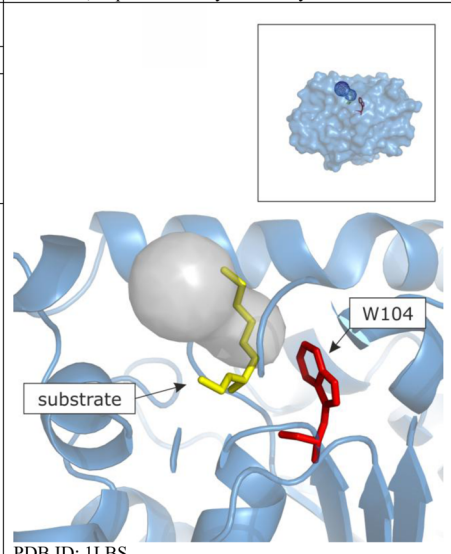
Table 3. continued

48	**Lipase B**	3.1.1.3
Gate function (lipase from *Candida antarctica*) **GATE1** – Controls regiospecificity, controls length of the substrate		**Enzyme function** Hydrolyse triacylglycerols and a broad range of other substrates, important for asymmetric synthesis
Gate location **GATE1** – Bottom of the substrate tunnel		 PDB ID: 1LBS
Gate structural basis		Tunnel
	GATE1 Wl04 The stereospecificity pocket is defined by T42, S47 and W104 Pocket is buried under a surface helix and delimited by the side chain of Wl04 *R* enantiomer of butanoic ester fits well into the active site pocket after a small movement of the side chain of Wl04	Length
Gate engineering W104A, W104Q – change in substrate specificity **Gate creation in other lipases** Lipase from *Burkholderia cepacia* – mutation in bottleneck for increased enantioselectivity – the best double mutant L17S/L287I - 15-fold increased activity and a tenfold enhanced enantioselectivity *Candida rugosa* lipase 1 – mutation of amino acids in different position inside the tunnel – P246F, L413F, L410W, L410F/S300E, L410F/S365L – different chain length selectivity		Bottleneck
PDB: ***WT (Candida antarctica)*** – 1LBS, 3ICV, 3ICW, ***WT (Burkholderia cepacia)*** – 3LIP, ***WT (Candida rugosa)*** – 1LPO		Role: Access to active site
References: [86,190–192]		

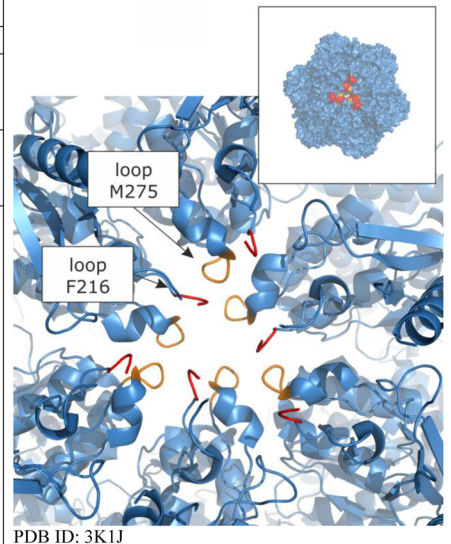
49	**Lon Protease**	3.4.21.53
Gate function **GATE1** – Controls the access of the substrate		**Enzyme function** ATP-dependent proteolysis
Gate location **GATE1** – At the entrance to an internal unfolding and degradation chamber		 PDB ID: 3K1J
Gate structural basis		Tunnel
	**GATE1** F216 loop	Length
	**GATE2** M275 loop	Bottleneck
Gate engineering F216A – lost almost all of the ATP-dependent proteolytic activity against a casein and the aromatic peptide		Role
PDB: ***WT*** – 3K1J		
References: [193]		

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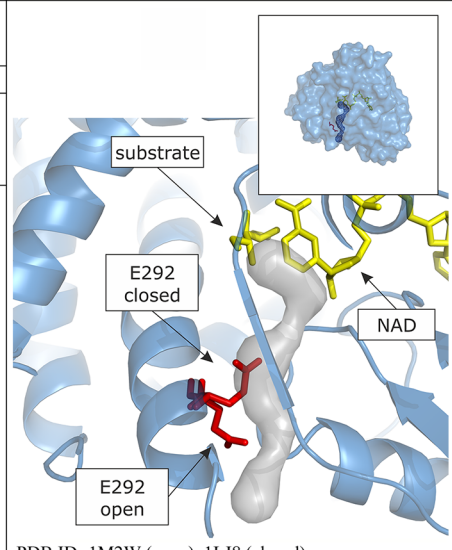
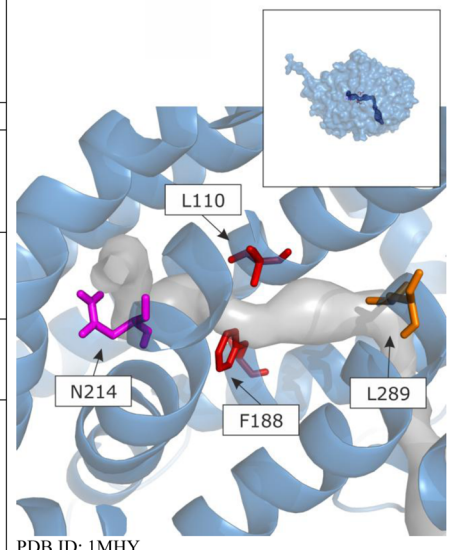
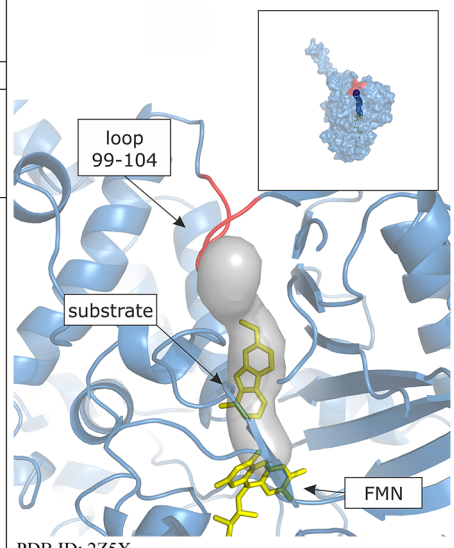
Table 3. continued

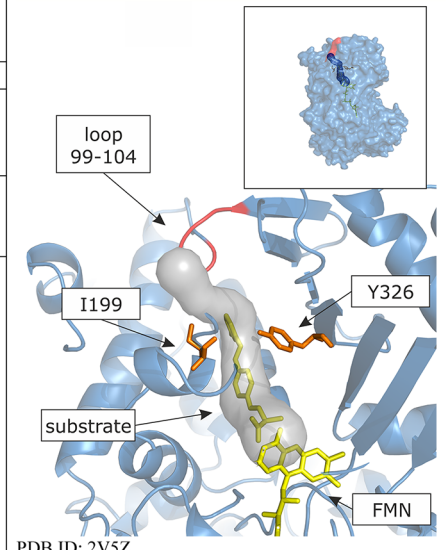
50	**Mannitol 2-Dehydrogenase**	1.1.1.67
Gate function GATE1 – E292 functions as a gate in water chain mechanism of proton translocation		**Enzyme function** Dehydrogenation of mannitol
Gate location GATE1 – Bottleneck of the tunnel connecting active site with protein surface		 PDB ID: 1M2W (open), 1LJ8 (closed)
Gate structural basis		
	GATE1 E292 may adopt two conformation open and closed	
Gate engineering E292A – 120-fold decrese in a rate of microscopic steps preceeding catalytic oxidation of mannitol		Tunnel
		Length
PDB: *WT* – 1LJ8 (closed), 1M2W (open)		Bottleneck
References: [194]		Role: Proton transfer

51	**Methane Monooxygenase Hydroxylase MMOH**	1.14.13.25
Gate function GATE1 – Controls transport of the substrate between cavities 1 and 2 GATE2 – Allows substrate to traverse the protein cavities GATE3 – Controls entrance of small substrates such as O_2, H_3O^+, CH_4		**Enzyme function** Converts hydrocarbon substrates either to alcohols or epoxides
Gate location GATE1 – Separates cavities 1 and 2 GATE2 – Separates cavities 2 and 3 GATE3 – One of the residue from active site is positioned at the interface between the cavity and the surface above the iron-coordinating residue		 PDB ID: 1MHY
Gate structural basis		
	GATE1 L110, F188 Conformation changes of L110 and F188 opens the access between the cavities 1 and 2, other residue involved T21	
	GATE2 L289 Moves to allow the substrate analogues to traverse between the cavities 2 and 3, other residues creating the bottleneck F109, V285 and Y291	
	GATE3 N214 Conformation changes – movement of N214 forms a deep crevice in the four-helix bundle	
Gate engineering		Tunnel
		Length
PDB: *WT* – 1MHY, 1XVG, 1XVF, 1XVE, 1XVB, 1XVC, 1XU5, 1XVD, 1XU3		Bottleneck
References: [53,74,195]		Role

dx.doi.org/10.1021/cr300384w | *Chem. Rev.* 2013, 113, 5871−5923

Table 3. continued

52	**Monoamine Oxidase A**	1.4.3.4
Gate function **GATE1** – Controls the access of the substrate		**Enzyme function** Oxidative deamination of biogenicamines and amine neurotransmitters (serotonin, dopamine and epinephrine)
Gate location **GATE1** – At the entrance to the active site cavity		 PDB ID: 2Z5X
Gate structural basis		Tunnel
	**GATE1** Loop 99-104 The loop movement is regulating access to the active site cavity and the loop flexibility is critical for opening the entry for substrates/inhibitors	Length
Gate engineering G110A – increase of K_m of 5-fold G110P – increase of K_m of 19-fold		Bottleneck
PDB: ***WT*** **–** 2Z5X, ***Mutant G110A*** **–** 2Z5Y		Role
References: [196,197]		

53	**Monoamine Oxidase B**	1.4.3.4
Gate function **GATE1** – Controls the access of the substrate **GATE2** – Controls the size of the active site cavity by separating or merging two smaller cavities		**Enzyme function** Oxidative deamination of biogenic amines and amine neurotransmitters (serotonin, dopamine and epinephrine)
Gate location **GATE1** – At the entrance to the active site cavity **GATE2** – Separates two internal cavities		 PDB ID: 2V5Z
Gate structural basis		Tunnel
	**GATE1** Loop 99-104 F103 side chain conformation movement is synchronized with the conformation changes of I199 residues (I199 open → F103 closed)	Length
	GATE2 I199, Y326 Movement of side chain residues separate or merge the entrance cavity and the substrate cavity	Bottleneck
Gate engineering I199A/Y326A – exhibits inhibitor binding properties more similar to those of monoamine oxidase A		Role
PDB: ***WT*** **–** 2V5Z, ***Mutant I199A/Y326A*** **–** 3ZYX		
References: [197,198]		

dx.doi.org/10.1021/cr300384w | *Chem. Rev.* 2013, 113, 5871−5923

Table 3. continued

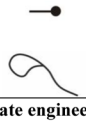
54	**Monooxygenase ActVAOrf6**	Not determined

Gate function
GATE1 – Controls access of the substrate, hydrogen bond donor and acceptor/proton gate
GATE2 – Controls opening of the narrow tunnel, can also control exit for H_2O, O_2, H_2O

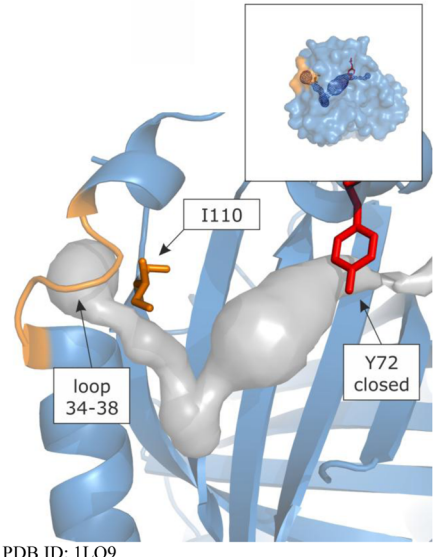
Enzyme function
Oxidation of a phenolic compound 6-deoxydihydrokalafungin at the C-6 position into the corresponding quinone dihydrokalafungin

Gate location
GATE1 – Entrance to the active site
GATE2 – Opposite side of the bound substrate

Gate structural basis

	GATE1 Y72 The residue possess two possible conformation that can act as a gate and act as hydrogen bond donor and acceptor
	**GATE2** I110 + loop (residues 34-38) Conformation changes close and open the narrow tunnel; gate can also control an exit path for H_2O

Gate engineering



PDB ID: 1LQ9

Tunnel		
Length		
Bottleneck		
Role	Proton transfer	Oxygen/water transport

PDB: ***WT*** **–** 1LQ9

References: [14,92]

55	**NADH Oxidase**	1.6.99.3

Gate function
GATE1 – Controls accessibility of the flavin ring and plays a crucial role during the catalytic cycle

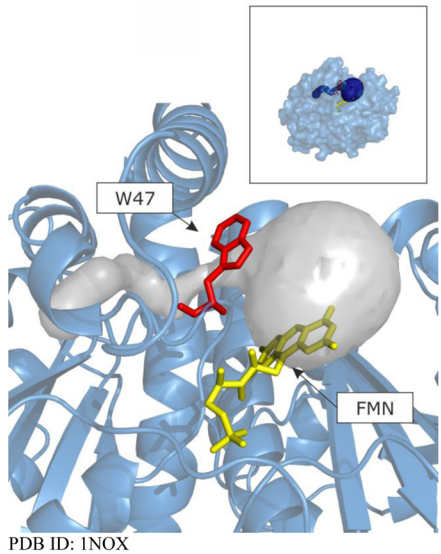
Enzyme function
Hydride transfer from NADH to the intrinsic flavin cofactor

Gate location
GATE1 – Above the active site

Gate structural basis

	GATE1 W47 and cofactor FAD Cofactor-assisted gating mechanism, W47 moves from the original position toward the pyrimidine section Closed conformation is stabilized by the hydrogen bonds between cofactor and peptide backbone Stabilization of the open form may have advantages during the initial steps of the substrate binding, it may slow down the product dissociation

Gate engineering



PDB ID: 1NOX

Tunnel	
Length	
Bottleneck	
Role	

PDB: ***WT*** **–** 1NOX

References: [97,199]

dx.doi.org/10.1021/cr300384w | *Chem. Rev.* 2013, 113, 5871−5923

Table 3. continued

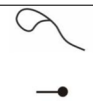
56	O-Acetylserine Sulfhydrylase Cysteine Synthase	2.5.1.47

Gate function
GATE1 – Controls access to the active site
GATE2 – Controls access to the active site

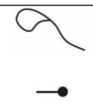
Enzyme function
Production of cysteine from the O-acetyl-serine intermediate

Gate location
GATE1 – Bottleneck of tunnel 12–20 Å from the active site cavity
GATE2 – Bottleneck of tunnel 8–12 Å from the active site cavity

Gate structural basis

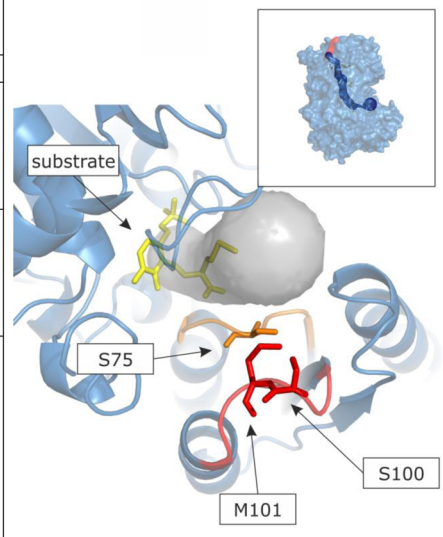


GATE1
Motion of side chains in addition to backbone movements M101 or S100 on the loop of the upper domain



GATE2
Motion of side chains in addition to backbone movements S75 on the Asn-loop for the inner gate

Gate engineering



PDB ID: 1Z7Y

Tunnel	
Length	~ 28
Bottleneck	
Role	Access to the active site

PDB: ***WT*** **–** 1Z7Y, 1Z7W

References: [200]

57	Oxidosqualene Cyclase SceOSC	5.4.99.7

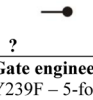
Gate function
GATE1 – Controls access of the substrate
?GATE2 – Controls access of the substrate

Enzyme function
Cyclization of the 2,3-oxidosqualene into lanosterol

Gate location
GATE1 – Bottleneck between tunnel and active site cavity
?GATE2 – Bottleneck between tunnel and active site cavity

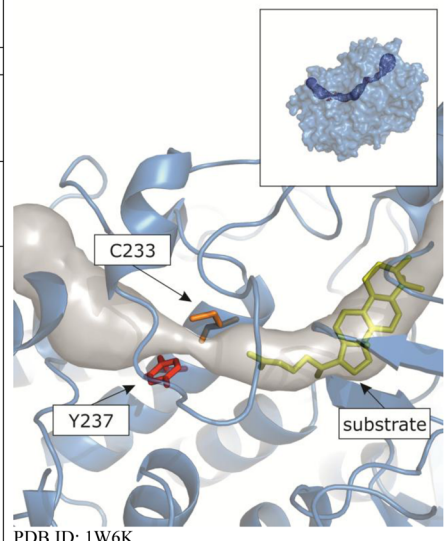
Gate structural basis

GATE1
Y239 (Y237 in *Hsa*OSC)
A rotation of the side chain stabilized by a hydrogen bond bridge with P228 could open the channel and enable the substrate to enter the active site



?GATE2
T235 (C233 in *Hsa*OSC)

Gate engineering
Y239F – 5-fold decrease in enzyme activity, mutation keeps the channel in a closed conformation
Y239A – 2-fold decrease in enzyme activity, mutation keeps the channel in a open conformation



PDB ID: 1W6K

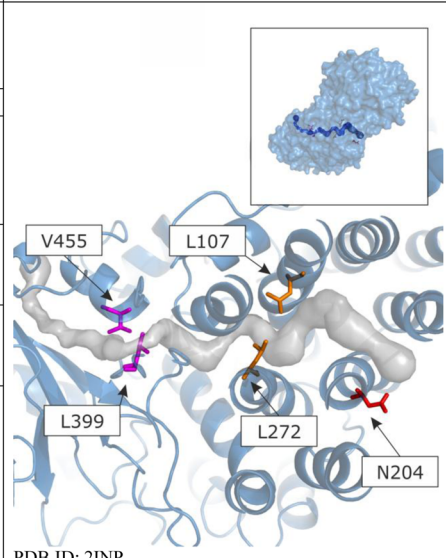
Tunnel	
Length	
Bottleneck	
Role	Connects active site cavity with bulk solvent

PDB: ***WT (HsaOSC)*** **–** 1W6K

References: [201]

dx.doi.org/10.1021/cr300384w | *Chem. Rev.* 2013, 113, 5871−5923

Table 3. continued

58	**Phenol Hydroxylase PHH**	1.14.13.7
Gate function GATE1 – Controls access to the active site GATE2 – Closes cavity 2 GATE3 – Closes cavity 2		**Enzyme function** Hydroxylates aromatic compounds
Gate location GATE1 – Entrance to the tunnel GATE2 – Between active site pocket and cavity 2 GATE3 – Between cavity 3 and cavity 2		
Gate structural basis		
	GATE1 N204 The residue shift is redox-dependent Helix E orients N204 away from the active site and opens the tunnel N204 in closed conformation forms a hydrogen bond with S72	
	GATE2 L107 and L272	
	GATE3 L399 and V455	
Gate engineering		PDB ID: 2INP
		Tunnel
		Length
PDB: ***WT*** – 2INP, 2INN		Bottleneck
References: [74,195]		Role: Substrate transport

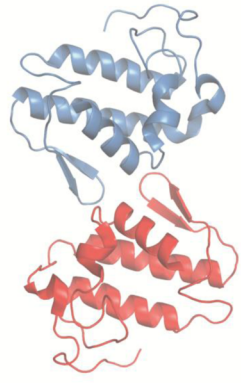
59	**Phospholipase A2**	3.1.1.4
Gate function GATE1 – Controls access to the interface and active sites		**Enzyme function** Catalyses the hydrolysis of acyl bonds in sn-3-phospholipids
Gate location GATE1 – Position of monomers		
Gate structural basis		
	GATE1 The dimer interface might act as a hinge The homodimer can be observed in open and closed conformations formed by different angle between monomers	
Gate engineering		PDB ID: 1CLP – open
		Tunnel
		Length
PDB: ***WT*** – 1CLP (open), 1PP2 (closed)		Bottleneck
References: [69,202,203]		Role

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Table 3. continued

60	Phosphatidylinositol-Specific Phospholipase C	4.6.1.13

Gate function
GATE1 – Controls access to the active site, controls product exit

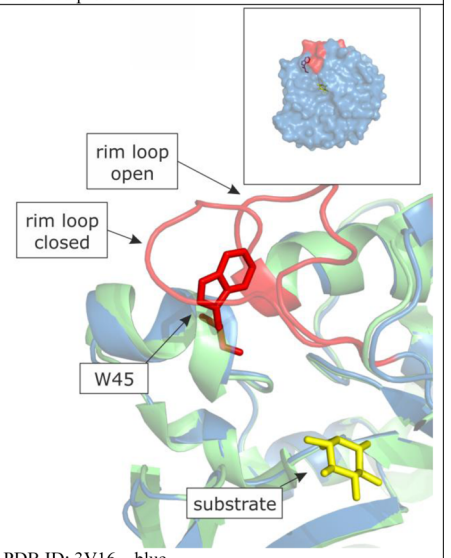
Enzyme function
Catalyses the cleavage of glycan-phosphatidylinositol anchored proteins

Gate location
GATE1 – Entrance to the active site

Gate structural basis

GATE1
Rim loop (residues 241-252) and W45
Rim loop undergoes the pH-dependent movement with a maximum backbone displacement of 9.4 Å between the acidic and basic forms
H258 serves as a anchoring residue holding F249 residue from a loop by π-cationic interaction
W45 closes the product exit pathway when the loop is in extended position

Gate engineering
H258Y – keeps the rim mobile loop in extended conformation



PDB ID: 3V16 – blue
PDB ID: 3V18 – light green

Tunnel	
Length	
Bottleneck	
Role	

PDB: ***WT*** – 3V16, 3V18, ***Mutants H258Y*** – 3V1H

References: [204]

61	Quercetin 2,3-Dioxygenase	1.13.11.24

Gate function
GATE1 – Controls access of small molecules into the active site
GATE2 – Controls access of small molecules into the active site

Enzyme function
Degradation pathway of flavonoids

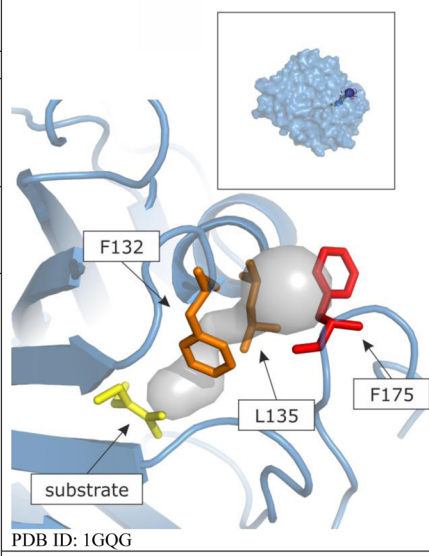
Gate location
GATE1 – Tunnel connecting the bulk solvent and the active site cavity
GATE2 – Tunnel connecting the bulk solvent and the active site cavity

Gate structural basis

GATE1
F175
Side-chain of F175 switches from the enzyme surface to the bulk and opens access to the cavity
This tunnel opening occurs as soon as the cavity is inflated by a sufficient number of water molecules (16–18)

GATE2
F132 and L135
Side-chains of F132 and L135 are structurally mobile enough to provide access for the entry of small molecules into the enzyme active site

Gate engineering



PDB ID: 1GQG

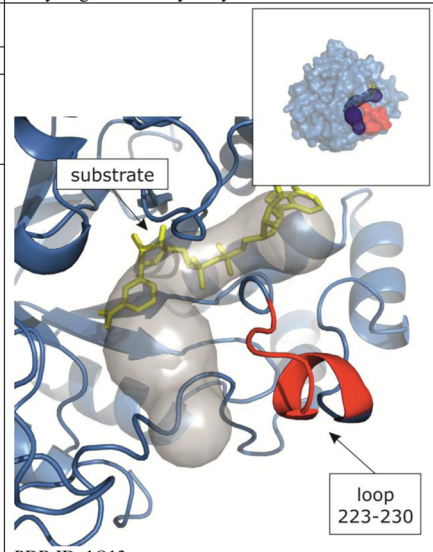
Tunnel	
Length	19
Bottleneck	1.6
Role	O_2 transport

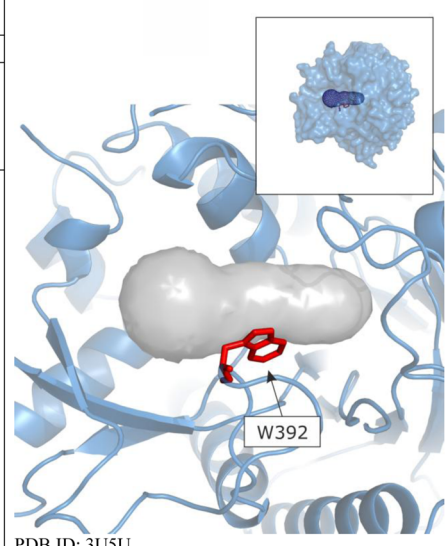
PDB: ***WT*** – 1GQG, 1JUH, 1H1I

References: [75]

dx.doi.org/10.1021/cr300384w | *Chem. Rev.* 2013, 113, 5871−5923

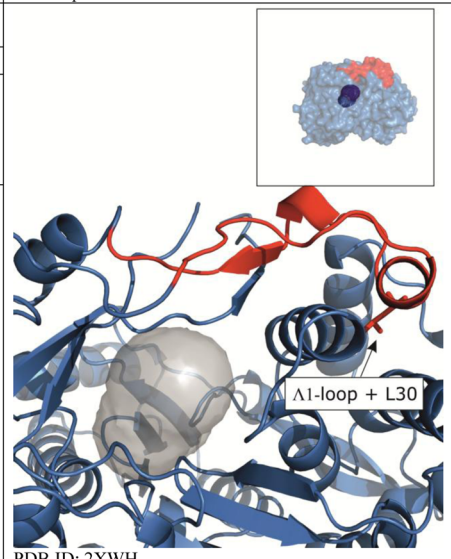
Table 3. continued

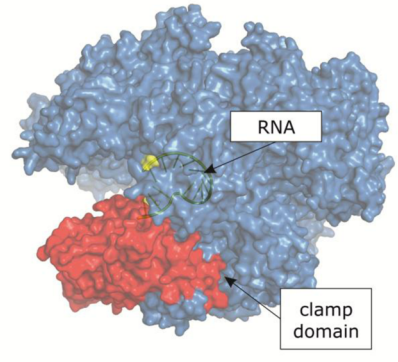
62	Rabbit 20a-Hydroxysteroid Dehydrogenase	1.1.1.149
Gate function **GATE1** – Protects cavity from the solvent in the absence of a steroid		**Enzyme function** Dehydrogenation of hydroxy steroid
Gate location **GATE1** – Loops making-up the active site cavity		
Gate structural basis		PDB ID: 1Q13
	**GATE1** Flexible loop B (residues 223–230) Movement of the loop partly controlled by the nature of Q230 Loops A and C mostly contribute in the pocket creation changing depth and size of the cavity	Tunnel
		Length
Gate engineering E230P – changes in the selectivity by reduced loop flexibility K274R – increases flexibility of loop by and eliminating K274–E227 contact, high k_{cat} for NADPH-dependent reduction of xylose		Bottleneck
		Role: Substrate steroid binding and orienting towards cofactor NADP(H)
PDB: ***WT*** **–** 1Q5M, 1Q13, ***WT (AKR5H1)*** **–** 2WZT 2WZM small loops		
References: [37,205–208]		

63	Raucaffricine O-β-Dglucosidaseglucosidase	3.2.1.125
Gate function **GATE1** – Ccontrols the access of the substrate		**Enzyme function** Hydrolyses alkaloid raucaffricine to aglyconevomilenine
Gate location **GATE1** – At the entrance to the active site cavity		
Gate structural basis		PDB ID: 3U5U
	GATE1 W392 Conformation of W392 is controlled by S390	Tunnel
		Length
Gate engineering S390G – leads to more flexible conformation of W392 F485W – results in more fixed conformation of W392		Bottleneck
		Role
PDB: ***WT*** **–** 4A3Y, ***Mutants E186Q*** **–** 3U5U, 3U57, 3U5Y		
References: [209]		

dx.doi.org/10.1021/cr300384w | *Chem. Rev.* 2013, 113, 5871–5923

Table 3. continued

64	RNA-dependent RNA polymerase	2.7.7.48
Gate function **GATE1** – Controls enzyme activity		**Enzyme function** RNA replication
Gate location **GATE1** – Surface loop		 PDB ID: 2XWH
Gate structural basis		Tunnel
	**GATE1** Λ1-loop + L30 Λ1-loop is involved in keeping the enzyme in a closed conformation, makes interaction with L30 (closed conformation is active, open is inactive)	Length: 19
Gate engineering **L30R** and **L30S** – open conformation **R222E** – exhibited 2-fold reduction in activity, positively charged residue inside the tunnel **K151E** – activity was 7–10-fold higher at 21 °C, and 2–3-fold higher at 37 °C, positively charged residue near the tunnel entrance		Bottleneck
PDB: ***WT*** **–** 2XWH, 2XXD, 3MWV		Role: NTP transport
References: [210]		

65	RNA Polymerase	2.7.7.6
Gate function **GATE1** – Controls the access to the DNA-binding tunnel		**Enzyme function** RNA synthesis
Gate location **GATE1** – Entrance to the DNA binding tunnel		 PDB ID: 1I6H
Gate structural basis		Tunnel
	**GATE1** Clamp domain may open to permit entry of promoter DNA during initiation, close to establish the tight grip on DNA during elongation and then open again to allow release of DNA during termination	Length
Gate engineering		Bottleneck
PDB: ***WT*** **–** 2O5J, 1I6H, 1I6V		Role
References: [66,211]		

dx.doi.org/10.1021/cr300384w | *Chem. Rev.* 2013, 113, 5871–5923

Table 3. continued

66	Toluene-4-Monooxygenase T4MO	1.14.13

Gate function
GATE1 – Controls access to the active site, controls selectivity
GATE2 – Controls access to the tunnel
GATE3 – Protects cavity from access of the solvent

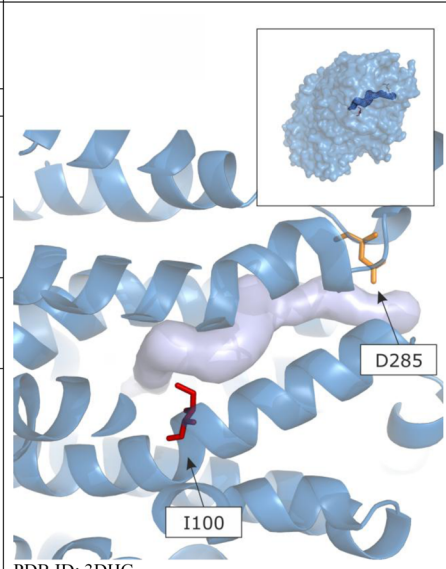
Enzyme function
Hydroxylates toluene primarily at the para position to cresol

Gate location
GATE1 – Boundary between the active site pocket and the tunnel
GATE2 – Tunnel entrance
GATE3 – Cavity entrance

Gate structural basis

—●	**GATE1** I100 Side chain rotation
—●	**GATE2** D285 Steric blocking by negatively charged residue D285
●— —●	**GATE3** R60, E64, E75, R69 Interactions between E64 and R60 and between E75 and R69, ionic gate for the cavity

Gate engineering
D285I, D285Q – improves oxidation of bulky substrates (11-fold)
D285S – improves oxidation of styrene (1.7-fold)
I100A – improves oxidation of bulky substrates (35-fold)
I100G – improves oxidation of methyl-p-tolyl sulphide (11-fold)



PDB ID: 3DHG

Tunnel	
Length	
Bottleneck	
Role	Substrate binding cavity

PDB: ***WT*** – 3DHG, ***WT (T4moC)*** – 1VM9, ***WT (T4moD)*** – 1G10

References: [61,105]

67	Toluene-Ortho-Xylene Monooxygenase ToMO	1.14

Gate function
GATE1 – Controls access to the active site, controls selectivity
GATE2 – Open pore allows access to the diiron centre for substrate (maybe also dioxygen or protons)

Enzyme function
Catalyses hydroxylation of aromatics, oxidize benzene to phenol, catechol and trihydroxybenzene

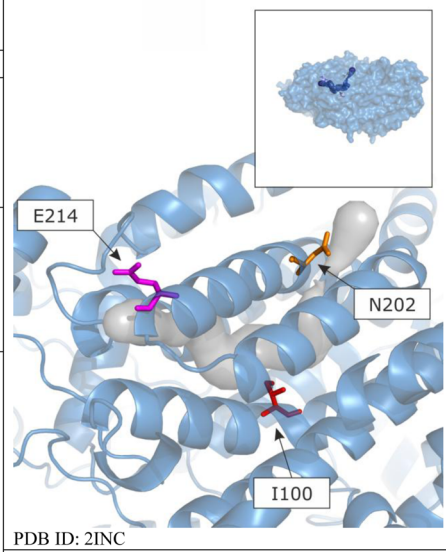
Gate location
GATE1 – Border of the pocket near Fe atoms – entrance to active site
GATE2 – Entrance to the tunnel

Gate structural basis

—●	**GATE1** I100 Residues H96, I100, T201, F205, and F196 border the pocket – I100 is an analogue of L110 (gate) in MMOH Ile100 contributes to defining the hypothetical para site, but it is also at the boundary between the active site pocket and the tunnel which connects the pocket to the surface of the protein
—●	**GATE2** N202 The conserved tunnel is gated by N202. The residue shifts in a redox-dependent manner. Its side chain is oriented away from the active site in the oxidized form and points inward in the reduced or Mn(II) reconstituted forms of the hydroxylase. This motion correlates with the carboxylate shift that occurs upon reduction of the dimetallic centre.

Gate engineering
I100A, I100W, F205L, A107V, A107I – decreased or removes activity

Creation of new **GATE3**
E214 – hot spot for new gate creation localized on helix E at the entrance to the tunnel 23 Å from the active site, mutation of those residue regulates access to the tunnel (E214A, E214G, E214V, E214W, E214F, E214Q, E214P)
E214G – 15-fold improvement for p-nitrophenol oxidation



PDB ID: 2INC

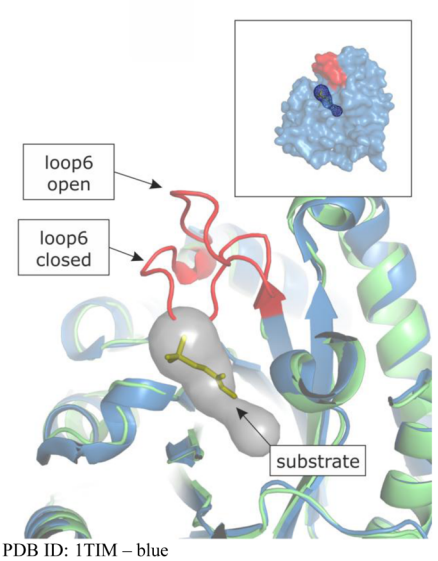
Tunnel	
Length	30-35
Bottleneck	
Role	Substrate and product transport

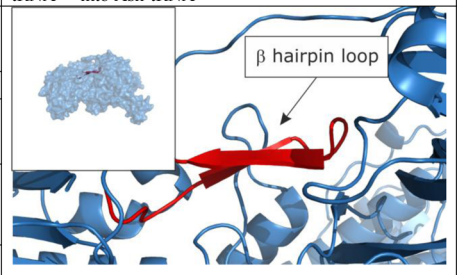
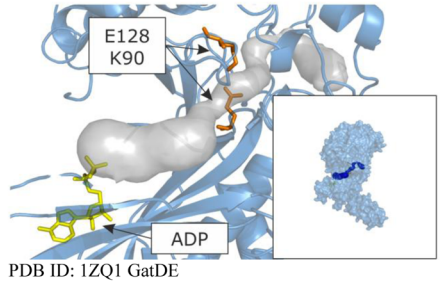
PDB: ***WT*** – 2INC, 1T0Q

References: [74,91,101,195,212,213]

dx.doi.org/10.1021/cr300384w | *Chem. Rev.* 2013, 113, 5871−5923

Table 3. continued

68	**Triosephophate Isomerase**	5.3.1.1
Gate function **GATE1** – Controls access of the water to the active site		**Enzyme function** Catalyses isomerisation D-glyceraldehyde 3-phosphate to dihydroxyacetone phosphate
Gate location **GATE1** – Entrance to active site		 PDB ID: 1TIM – blue PDB ID: 1TPH – light green
Gate structural basis		
	**GATE1** Loop 6 (residues 166 to 176). Residues 169 – 173 move as a rigid body, which position is controlled by flexible three residues N-terminal and C-terminal hinge regions In closed conformation loop sequesters the enzyme reaction from solvent. Closed conformation is stabilised by conserved anchoring residues from loop 7	
Gate engineering P(166)VW-AIGTG-KTA to P(166)GG-AIGTG-GGG mutant – k_{cat} decreases 2500-fold Y208F – k_{cat} decreases 2400-fold Y208T – k_{cat} decreases 200-fold		**Tunnel**
		Length
PDB: ***WT*** – 1TIM (open), 1TPH (closed)		**Bottleneck**
References:[77,78,214–216]		**Role**

69	**tRNA-Dependent Amidotransferase GatDE and GatCAB**	6.3.5
Gate function **GATE1** – Protects active site from access of the solvent, avoids wasteful release of ammonia into solution **?GATE2** – Controls ammonia transport		**Enzyme function** Conversion of Glu-tRNA^{Gln} into Gln-tRNA^{Gln} or Asp-tRNA^{Asn} into Asn-tRNA^{Asn}
Gate location **GATE1** – β hairpin loop surrounding asparaginase active site (in GatDE) **?GATE2** – Inside ammonia tunnel (in GatCAB)		
Gate structural basis		
	**GATE1** GatDE β hairpin loop Dβ7-Dβ8, residues 100–118 in GatD, G100, the first glycine in the strictly conserved GGT motif, active conformation substrate in contact with T102	
	GATE2 GatCAB in *A. aeolicus* (*S. aureus*) E128 (E125), K90 (K88) E128 (E125) side chain can blocks the tunnel via a salt bridge with K90 (K88)	
Gate engineering		 PDB ID: 1ZQ1 GatDE PDB ID: 3H0L GatCAB
		Tunnel GatDE / GatCAB
		Length GatCAB: 35
PDB: ***WT (GatDE)*** **–** 1ZQ1, ***WT (GatCAB)*** **–** 3H0L, 3H0M, 3H0R		**Bottleneck**
References: [155,217–219]		**Role** Ammonia transport

dx.doi.org/10.1021/cr300384w | *Chem. Rev.* 2013, 113, 5871−5923

Table 3. continued

70	**Tryptophan Synthase**	4.2.1.20

Gate function
GATE1 – Creates the tunnel and protects substrate binding cavity from the access of the solvent
GATE2 – Controls transport of the indol
GATE3 – Controls access to the subunit β and activates the β active site

Enzyme function
Synthesis of L–tryptophan; subunit α cleavage of 3-indole-D-glycerol 3-phosphate; subunit β pyridoxal phosphate dependent condensation of indole with L-serine

Gate location
GATE1 – Surface loop from subunit α at the border between the subunits β and α
GATE2 – Tunnel wall in subunit β
GATE3 – Subunit β

Gate structural basis

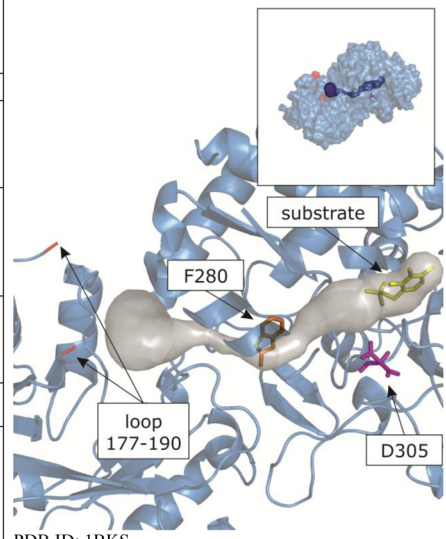
GATE1
Loop L177 - A190
A disordered surface loop in the subunit α after substrate binding becomes ordered and clamps down over the active site, isolating this region from solvent

GATE2
F280, Y279
The residue F280 can adopt alternative conformations in closed interact with C170 in open one it adopts Y279 position, Y279 moves toward the subunit α and interacts with part of the flexible loop-2 residues (54-61) of the subunit α

GATE3
D305
In the closed conformation D305 creates the H-bonded salt bridge with R141with the associated H-bonding network involving S197 and S199

Conformation changes are initiated by binding IGP - the allosteric communication between the two sites that results in full coupling of the reaction at the subunits α and β

Gate engineering
E49F, G51L, D60Y – decreases activity (2-fold)
F280C, F280S – increases transport
C170W, C170F – chemical modification of C170 (C170-NEM, C170-MMTS), obstructs the tunnel and accumulates indole intermediate



PDB ID: 1BKS

Tunnel	
Length	25 – 30
Bottleneck	
Role	Indol transport between two active sites

PDB: ***WT*** **–** closed 3CEP, open conformations: 1BKS, 1KFK, 1KFJ, 1TTP, 2CLL, 2CLM, 2CLO

References: [47,71,102,220,221]

71	**Undecaprenyl-Pyrophosphate Synthase**	2.5.1.31

Gate function
GATE1– Controls length of the final product

Enzyme function
Condensation reactions of isopentenyl pyrophosphate with allylic pyrophosphate to generate linear isoprenyl polymers

Gate location
GATE1 – Loop closing the entrance to the tunnel

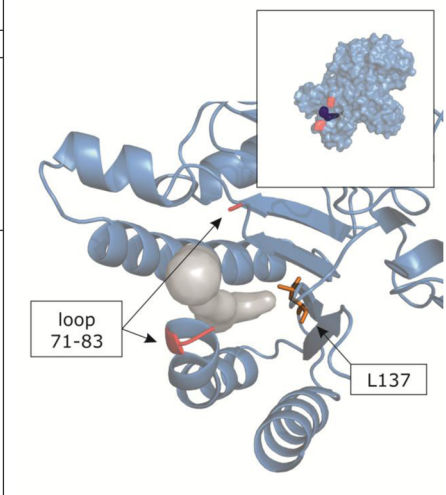
Gate structural basis

GATE1
71 – 83 loop
The helix α3 in open tunnel conformation is kinked by ~30° at E96, and the helix α3 in closed tunnel conformation is kinked by ~45° at A92.
Reaction starts with closed conformation → when the chain length reaches C55, the dimethyl end is stopped at the end of the tunnel → α3 helix move away from the closed position into the open position (71 – 83 loop) → fully synthesized C55 long product can exit easily through the open gate

Gate engineering
*GATE2
L137A – removes the floor of the tunnel and allows formation of a longer chain length products (normal length C55), bulky side chain of L137 serves to block further elongation of undecaprenyl-pyrophosphate

A69L – results in long lived accumulation of a short chain intermediate C30 final product C55
A143V – similar to wild type, rate 3-fold lower

S71A, N74A, or R77A – decreases in k_{cat} values (25–200-fold)
W75A – increases in K_m for farnesyl pyrophosphate (8-fold)
E81A, S71A – increases in K_m for isopentenyl pyrophosphate (22–33-fold)



PDB ID: 1JP3

Tunnel	
Length	30
Bottleneck	
Role	Active site cavity

PDB: ***WT*** **–** 1JP3

References: [27]

substrate binding since it is believed to slow down product dissociation.[22,97] Other gates of this type have been reported in 3-hydroxybenzoate hydroxylase,[98] 4-hydroxy-2-ketovalerate aldolase/acylating acetaldehyde dehydrogenase,[96] and cholesterol oxidase type I[57] and type II.[99] Moreover, cofactors themselves can also function as gates. The FAD cofactor of digeranylgeranylglycerophospholipid reductase has two different conformations, referred to as the "in" and "out"

dx.doi.org/10.1021/cr300384w | Chem. Rev. 2013, 113, 5871–5923

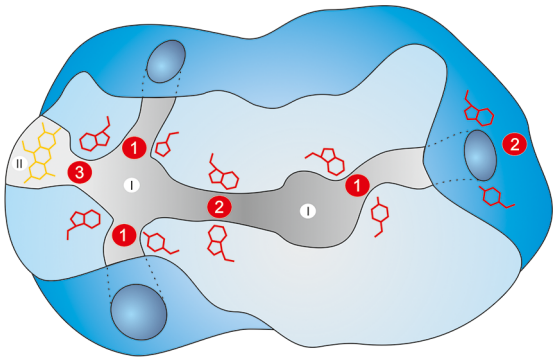


Figure 6. Locations of gates within a protein structure. Schematic representation of an enzyme with two active sites connected by a tunnel (I), a cofactor cavity (II), and multiple access tunnels. Gating residues in red may be located at the entrance to the active site (1), at the entrance or the bottleneck of the tunnel (2), and between the active site cavity and the cofactor cavity (3).

conformations. In the "in" conformation, the tunnel is blocked by FAD. To open the tunnel, FAD has to adopt the "out" conformation.[100]

Gates are most commonly located at the tunnel entry and the tunnel bottleneck (51%). This is to be expected because the bottleneck represents the narrowest point of the tunnel, and its diameter often dictates the tunnel's permeability. Another common location is the entrance to the active site cavity (28%). Gates at the entrance of the cofactor cavity are less common (5%), which is not surprising since not all enzymes have a cofactor cavity. In 16% of the cases studied, the gate was not located within any of these functional regions (Figure 7).

5. ENGINEERING OF GATES

The average rate of evolution of the gating residues in the cytochrome P450 family is significantly greater than that for the protein sequence as a whole.[59] The high rate of evolution at the gating residues suggests that gate engineering may be an attractive alternative to other rational enzyme design strategies. This idea is supported by a few observations: (i) the gates are often spatially separated from the active site, and so mutations at the gating residues should not be deleterious to protein function, (ii) the opening and closing of the access pathways can affect ligand exchange and thus enzyme activity and selectivity, and (iii) gate modification can modulate the solvent's ability to access the active site, which in turn affects solvation and stabilization of the transition state and also product release. In the following section, we describe selected cases in which an enzyme's catalytic properties have been successfully altered by modifying its gates.

5.1. Gate Modification

Gates can be modified by substitution of the gating residues, hinge residues, or anchoring residues depending on the nature of the gate in question. Modification of the gating residues has been shown to change the selectivity and activity of toluene-*o*-xylene monooxygenase, with the E214G mutation improving oxidation of *p*-nitrophenol by a factor of 15.[101] A similar improvement in overall activity was achieved in a lipase from *Burkholderia cepacia* by the mutations L17S + L287I. This double mutant also exhibited a 10-fold increase in enantioselectivity compared to the wild-type enzyme.[86] The T78F or P76F mutations in imidazole glycerol phosphate synthase override some of the control exerted by the wild-type gate and block the passage of ammonia through the tunnel.[38] In NiFe hydrogenases, mutations of the V74 and V74 + L122 residues changes the rates of transport for H_2, CO, and O_2, thereby modulating the overall rate of reaction.[21]

Gates that incorporate secondary structure elements are dependent on hinge and anchoring regions. It has been demonstrated that modification of the hinge region can change an enzyme's activity and selectivity. Notably, the Q230P mutation in rabbit 20A-hydroxysteroid dehydrogenase decreases the flexibility of a key loop and thereby changes its selectivity.[37] Similarly, access to the active site in the HIV-1 protease is controlled by two β-turn flaps.[89] Results from Brownian dynamics simulations suggest that the G48V + V82A or I84V + L90M mutations in this enzyme reduce the likelihood that the active site will be exposed at any given point in time from 14% in the wild type to 2% in the mutants.[89] The importance of the anchoring residues in the HIV-1 protease was demonstrated by the F53L mutation, which generates a semiopen conformation due to removal of the stabilizing F53–I50 interaction.[89]

5.2. Gate Removal

Removing gates typically increases substrate and product exchange rates but also allows more extensive access of water molecules to protein tunnels and cavities. The overall effect of gate removal is therefore equal to the combined effects of these two processes. Gate deletion has been shown to increase the rate of substrate binding to tryptophan synthase.[102] The F280C and F280S mutations both increased the rate of indole binding

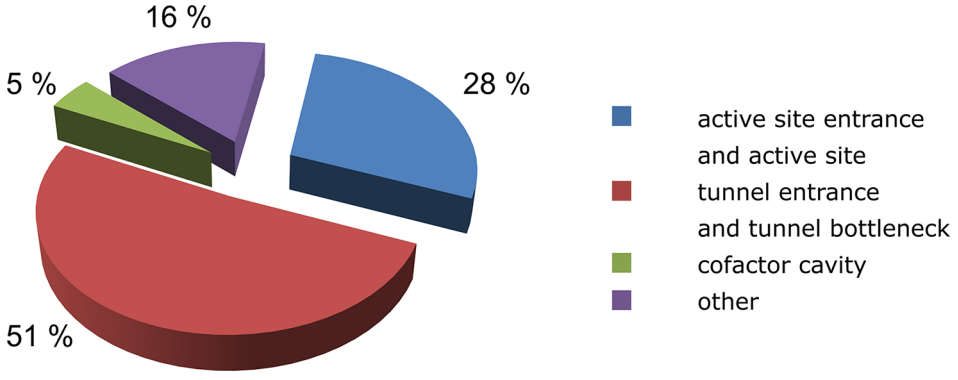


Figure 7. Distribution of gate locations within protein structures based on analysis of 71 proteins with 129 gates. Detailed description of the analyzed proteins is provided in Table 3.

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by a factor of 2. Similarly, the T78A mutation allowed ammonia to rapidly pass through the tunnel in imidazole glycerol phosphate synthase.[38] The R239A mutation in the cyclase caused a 1000-fold decrease in the enzyme's k_{cat}/K_m value and decoupling of the reaction.[38] This dramatic change in enzyme catalytic efficiency was attributed to creation of a new route for ammonia release. A similar leakage of ammonia was caused by the G359F and G359Y mutations in carbamoyl phosphate synthetase.[103,104] Negative consequences of gate removal were also observed for the FabZ-β-hydroxyacyl-acyl carrier protein dehydratase (HpFabZ),[54] in which the Y100A mutation leaves the active site completely exposed to the bulk solvent. As a result, the acyl carrier protein binds to the HpFabZ Y100A mutant much more strongly than to the wild-type HpFabZ, decreasing the mutant enzyme's activity by more than 50% due to the very slow dissociation of the acyl carrier protein.

In some cases, gate removal enables bulky substrates to access the active site cavity. Mutations D285I and D285Q in toluene-4-monooxygenase improved its ability to oxidize the large and bulky substrates 2-phenylethanol and methyl *p*-tolyl sulfide by factors of 8 and 11, respectively, while the D285S mutation improved the rate of styrene oxidation 1.7-fold.[105] The L137A mutation in undecaprenyl-pyrophosphate synthase removed the bottom of the tunnel in this enzyme, allowing formation of products with longer chain lengths.[27] A similar situation was encountered in type III polyketide synthases from *Aloe arborescens* PCS, in which the M207 residue controls the number of condensations of malonyl-CoA.[106] The M207G mutation opened a connection between the tunnel and two hidden pockets located behind the active site, resulting in formation of extended products. Further product elongation was achieved with the triple mutant F80A + Y82A + M207G.

5.3. Gate Insertion

To best of our knowledge, there have been no reports of an intentional introduction of a new gate into an enzyme structure. However, there have been studies in which an access tunnel was systematically modified with multiple substitutions, and it is reasonable to expect that some of these mutations might have created new gates. More research will clearly be needed to confirm this expectation. To verify successful intentional insertion of a new gate into a protein structure, it would be necessary to confirm the existence of both the open and the closed conformations at a position where previously only a single conformation could be adopted.

Site-directed mutagenesis targeting specific residues at various positions along the access tunnel of *Candida rugosa* lipase has been used to alter the acceptable substrate chain length for this enzyme. The mutants for which this was observed were P246F, L413F, L410W, L410F + S300E, and L410F + S365L.[107] We note that the aromatic residues F and W, which are common in wing and swinging door gates, were introduced in each of these variants.

In another study, the residue L177 that is located near the entrance to the access tunnel of the haloalkane dehalogenase LinB from *Sphingobium japonicum* UT26 was substituted with all of the natural amino acids, yielding 19 mutants with significantly altered substrate specificity and activity.[108] Preliminary computational analyses of these variants using molecular dynamics revealed that the two residues possessing a single aromatic ring (F and Y) exhibited large fluctuations, as might be expected for gating.

Residue A217 is located at the entrance to the tunnel in the epoxide hydrolase EH from *Aspergillus niger* M200. This residue was substituted with C, E, G, L, P, Q, R, T, and V, and the effect of each mutation on the enzyme's activity and enantioselectivity was studied.[26] The mutants exhibited different enantioselectivity and activity relative to the wild type. For instance, the activity of the A217G mutation toward allyl glycidyl ether was lower than that of the wild type by a factor of 33, whereas the A217V mutation increased activity toward this substrate 6.6-fold.

Residues I135, W141, C176, V245, L246, and Y273 are positioned close to the entrance to the main and side tunnels of the haloalkane dehalogenase DhaA from *Rhodococcus rhodochrous* NCIMB 13064. These residues were simultaneously permuted in an attempt to improve this enzyme's activity against 1,2,3-trichloropropane. The most successful mutant, which featured the I135F, C176Y, V245F, L246I, and Y273F substitutions, showed 26-fold greater activity toward the target substrate than did the wild type.[109] In this mutant, three aromatic residues were introduced in place of aliphatic ones in the vicinity of the tunnels. Computational analysis of product release from the mutant suggests that substitutions introduced a transient rather than permanent structural feature and gating residues prevented access of water to the active site.[110] Crystallographic analysis of the mutant revealed two distinct conformations for the Y176 side chain.[110]

6. CONCLUSIONS

This review highlights the importance of gates in enzymes. Gates play vital roles in controlling the catalytic activity and selectivity of enzymes and are more common in protein structures than is generally thought. In particular, gates control substrate access to the active site and product release, prevent or restrict solvent access to specific regions of the protein, and can synchronize processes occurring in distinct parts of the enzyme. Our literature survey of 129 gates in 71 enzymes revealed a large variety of systems with sophisticated structures. We presented a rigorous definition of gates and established a new scheme for their classification. The large number of inspected cases allowed us to build a catalogue of gates assigned to six distinct classes—wings, swinging doors, apertures, drawbridges, double drawbridges, and shells—with three different functions and three distinguishable locations. We also presented summary statistics that give a preliminary overview of the propensity of specific amino acid residues to occur in particular gate classes. The proposed classification scheme can be easily extended and updated but even in its present form can provide guidance for analysis and engineering of gates in biomolecular systems.

The biochemical relevance and specific location of gates within protein structures make them attractive targets for protein engineering. Attempts to rationally redesign gates typically involve computer-assisted gate identification followed by modification using focused directed evolution. This approach is compatible with a recent trend in protein engineering that stresses construction of small and smart libraries. Gate modification and deletion have been demonstrated in numerous cases, but the intentional insertion of new gates remains a challenge. Convenient methods for identifying gates in protein structures are essential prerequisites for their engineering. In silico, this can be achieved by coupling the software tools developed for describing pathways to tools developed for study of protein dynamics. Of the available

experimental techniques, NMR spectroscopy is particularly suitable for analysis of highly dynamic protein structures and can be expected to play an indispensable role in the study of gate dynamics at the atomic level. The field would also benefit from development of new experimental techniques for monitoring the passage of ligands through the protein pathways. One day it will be possible to control the catalytic properties of enzymes by rational engineering of their gates. To achieve this goal, we have to learn how gates evolved, how they interact with the other parts of the protein structure as well as with the ligand and solvent molecules, and how they fulfill their biological functions.

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Notes

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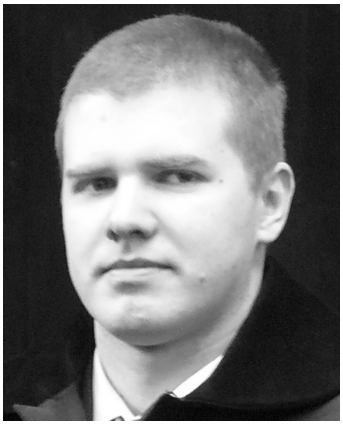

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